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Priprava inkluzijskih teles z visokim deležem pravilno zvitega prekurzorja heterolognega proteina

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Erik Vrenko
direktor

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Priprava inkluzijskih teles z visokim deležem pravilno zvitega prekurzorja heterolognega proteina

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Priprava inkluzijskih teles z visokim deležem pravilno zvitega prekurzorja heterolognega proteina

Področje tehnike

Predloženi izum se nanaša na nov postopek pridobivanja biološko aktivnega granulocitne kolonije stimulirajočega dejavnika (G-CSF), ki poteka na tak način, da se že v inkluzijskih telesih nahaja pravilno zvit prekurzor G-CSF.

G-CSF uvrščamo med kolonije stimulirajoče dejavnike, ki regulirajo diferenciacijo in proliferacijo hematopoetskih celic sesalcev. Imajo odločilno vlogo pri tvorbi nevtrofilcev, zato so primerni za uporabo v medicini na področju hematologije in onkologije.

Za klinično uporabo sta danes na trgu dve obliki: lenograstim, ki je glikoziliran, in ga pridobivajo z ekspresijo v sesalski celični liniji, ter filgrastim, ki je neglikoziliran, in ga pridobivajo z ekspresijo v bakteriji *Escherichia coli* (*E. coli*).

Bistvo predloženega izuma

Bistvo predloženega izuma je postopek pridobivanja biološko aktivnega G-CSF, ki vključuje vodenje biosinteze G-CSF na tak način, da se izloča pravilno zvit prekurzor G-CSF že v inkluzijskih telesih.

Tak način vodenja biosinteze vključuje enega ali več parametrov, ki so izbrani iz skupine, ki sestoji iz temperature gojenja, načina indukcije, principa vodenja fermentacije, sestave gojišča in ko-ekspresije pomožnih proteinov.

Postopek pridobivanja biološko aktivnega G-CSF, ki je predmet izuma, dodatno vključuje spiranje in raztapljanje inkluzijskih teles ter omogoča direktno izolacijo biološko aktivnega proteina brez uporabe denaturantov ali postopka renaturacije.

Stanje tehnike

Humani granulocitne kolonije stimulirajoči faktor (hG-CSF) uvrščamo med hematopoetske rastne dejavnike in ima odločilno vlogo pri tvorbi nevtrofilcev. Za

klinično uporabo sta danes na trgu dve obliki: lenograstim, ki je glikoziliran in ga pridobivajo s sesalsko celično linijo CHO (Holloway C.J. (1994) *Eur J Cancer* 30A Suppl 3:S2-S6., EP 169566), ter filgrastim, ki je neglikoziliran in ga pridobivajo z ekspresijo v bakteriji *E. coli* (EP 237545).

Pri intracelularni produkciji heterolognega G-CSF v bakteriji *E. coli* se protein vedno izloča v obliki netopnih inkluzijskih teles. Tudi pri poskusih sekrecije v periplazmo *E. coli* se je G-CSF ali izločil v obliki inkluzijskih teles (Lei S.P s sodelavci (1987) *J Bacteriol* 169:4379-4383; Chung B.H. s sodelavci (1998) *J.Ferment.Bioeng.* 85:443-446.) ali pa ni nobenih podatkov o biološki aktivnosti tako produciranega G-CSF (Jeong K.J. in Lee S.Y. (2001) *Protein Expression and Purification* 23: 311-318).

Iz navedenega je jasno, da pri skoraj vseh doslej opisanih poskusih izolacije G-CSF iz bakterije *E. coli* izhajajo iz inkluzijskih teles.

Opisanih je tudi nekaj primerov, kjer dobimo inkluzijska telesa v kvasovkah, vendar o G-CSF nikjer ne poročajo.

Za pridobivanje pravilno zvitih biološko aktivnih proteinov iz netopnih inkluzijskih teles je potrebno inkluzijska telesa izolirati iz celice, jih sprati, raztopiti in nato izvajati *in vitro* renaturacijo. *In vitro* renaturacija predstavlja dodatne stopnje v procesu industrijske izolacije proteinov.

Postopki pridobivanja rekombinantnih proteinov iz inkluzijskih teles vključujejo zato lizo in razbitje celic, čemur sledi centrifugiranje. Pelet, ki vsebuje velik delež inkluzijskih teles, se navadno spere z detergenti. V primeru izolacije G-CSF navajajo kot primerno spiranje inkluzijskih teles z 1 % deoksiholatom (Zsebo K.M. s sodelavci (1986) *Immunobiology* 172:175-184. EP237545; US5849883) ali z raztopino 1,0 M NaCl z dodatkom 0,1% Tween 80 (Gelunaite L. s sodelavci (2000) *J Chromatogr A* 904:131-143).

Naslednjo stopnjo v pridobivanju rekombinantih proteinov predstavlja raztapljanje inkluzijskih teles, kar zahteva navadno uporabo precej močnih denaturantov. Pri pridobivanju G-CSF je opisana uporaba 6 M GdHCl (Wingfield P s sodelavci (1988) *Biochem J* 256:213-218; Zink T. s sodelavci (1992) *FEBS Lett* 314:435-439.), 7 M uree pri pH 7,2 (Kuga T. s sodelavci (1989) *Biochem Biophys Res Commun* 159:103-111) ali 8 M uree pH 8,0 (Yamasaki M. s sodelavci (1998) *Biosci Biotechnol Biochem* 62:1528-1534.) 20 mM TrisHCl in dodatkov EDTA in DTT.

Kang S-H et al. so uspeli raztopiti inkluzijska telesa v 2 M urei v zelo alkalnem pH okoli 12 (Kang S.H. s sodelavci (1995) *Biotechnol.Lett.* 17:687-692). Podoben je postopek raztapljanja v 10 mM HEPES pufru s kratkotrajnim zvišanjem pH na 11,5 (Gelunaite L. s sodelavci (2000) *J Chromatogr A* 904:131-143.). Enako učinkoviti za raztapljanje so tudi močni detergenti v denaturirajočih koncentracijah npr. 1 % SDS z dodatki 5 % etanola v 50 mM TrisHCl pH 8.5 (Souza L.M. s sodelavci (1986) *Science* 232:61-65.). Večkrat uporabijo 2 % sarkozil (Lu H.S. s sodelavci (1992) *J Biol Chem* 267:8770-8777 Lu H.S. s sodelavci (1993) *Protein Expr Purif* 4:465-472.; Young DC s sodelavci (1997) *Protein Science* 6: 1228-1236; EP237545; US5849883) ali tudi samo 1% sarkozil (Zsebo K.M. s sodelavci (1986) *Immunobiology* 172:175-184). Raztapljanje inkluzijskih teles v večini primerov zahteva uporabo močnih denaturantov, ki so strupeni in s tem škodljivi okolju, saj predstavljajo resno onesnaženje okolja. Njihova uporaba je tudi neekonomična, saj varna odstranitev po končanem procesu predstavlja dodaten strošek in je časovno zamudna.

Čeprav so opisani nekateri primeri, kjer v prvi kromatografski stopnji ločujejo protein pod denaturirajočimi pogoji, npr. na C4 RP HPLC (Souza L.M. s sodelavci (1986) *Science* 232:61-65.; Zsebo K.M. s sodelavci (1986) *Immunobiology* 172:175-184; EP237545) ali z gelsko filtracijo (Wingfield P. s sodelavci (1988) *Biochem J* 256:213-218; US4999291), poteka v večini primerov kromatografska separacija po predhodni *in vitro* renaturaciji.

Po raztapljanju inkluzijskih teles v močnih denaturantih sledi *in vitro* renaturacija proteinov. *In vitro* renaturacijo G-CSF dosežejo z dializo proti pufru z manjšo koncentracijo denaturanta (Wingfield P. s sodelavci (1988) *Biochem J* 256:213-218), z dializo proti pufru brez denaturanta (Kuga T. s sodelavci (1989) *Biochem Biophys Res Commun* 159:103-111), v pufru z dodatkom 0.8 M arginina (Zink T. s s sodelavci (1992) *FEBS Lett* 314:435-439) ali z gelsko filtracijo (US4999291; D.C. Young et al. (1997) *Protein Science* 6: 1228-1236). Po raztapljanju v denaturirajočih koncentracijah sarkozila pa dosežejo oksidativno zvitje z dodatkom nizkih koncentracij $CuSO_4$ med dolgotrajnim mešanjem pri sobni temperaturi, čemur sledi odstranitev detergenta z močno bazičnim ionskim izmenjevalcem (US5849883). V primerih raztapljanja v ekstremno bazičnih pogojih se protein spontano renaturira z vzpostavitvijo nižjega pH (Kang S.H. s sodelavci

(1995) *Biotechnol. Lett.* 17:687-692). Vsi opisani postopki *in vitro* renaturacije so kompleksni in časovno zamudni. Dilucija v industrijskem merilu pa zahteva naprave velikih kapacitet, kar posledično pomeni večjo začetno investicijo (večji prostor, večje aparature...), pri obratovanju pa večje obratovalne stroške (energija, voda, pufri...).

Izboljšana *in vitro* renaturacija je bila dosežena pri fuzijskem proteinu, kjer je bil na N-terminusu dodan oligopeptidni hidrofilni podaljšek in cepitveno mesto za IgA proteazo (EP0500108).

V literaturi je bilo že večkrat opisano povečevanje deleža topnega proteina v citoplazmi (Weickert M.J. in sodelavci (1997), Appl. and Environmental Microbiology, 63:4313-4320; Bhandari P. in Gowrishankar J. (1997) J. Bacteriol., 179: 4403-4406; Zhang Y. s sodelavci (1998) Protein Expr. Purif., 12: 159-165; Thomas J.G. in Baneyx F. (1996) J of Biological Chemistry, 271:11141-11147; Kapust R.B. in Waugh D.S. (1999) Protein Science, 8: 1668-1674; Davis G.D. s sodelavci (1999) Biotech. and Bioeng. 65: 382-388), nikjer pa ni opisano, da bi se tudi G-CSF namesto v inkluzijskih telesih izločil v citoplazmi.

Ne v patentni ne v strokovni literaturi niso opisani pristopi, ki bi obravnavali povečanje deleža pravilno zvitega heterolognega proteina že v inkluzijskih telesih.

Prav v primeru G-CSF navajajo *in vitro* renaturacijo kot prvi pogoj in s tem edini možni način za pridobivanje biološko aktivnega proteina (Rudolph R. (1996) v *Protein Engineering: Principles and Practice* (Cleland JL and Craik CS eds) pp 283-298, Wiley-Liss, Inc., New York).

Opis slik

Slika 1: Sestava proteinov v inkluzijskih telesih

Metoda: poliakrilamidna gelska elektroforeza v prisotnosti natrijevega dodecil sulfata (SDS-PAGE) (4% zgoščevalni, 15% ločitveni gel, barvano s Coomassie brilliant blue).

Pogoji priprave inkluzijskih teles: temperatura gojenja 25°C, vodenje fermentacije po principu šaržnih fermentacij, indukcija z 0.4 mM izopropil-beta-D-tiogalaktopiranozidom (IPTG) pri $OD_{600nm} \approx 6.0$ oziroma z začetno indukcijo, spiranje z ohlajeno vodo ali ohlajenim 10 mM TrisHCl/pH=8.0 pufrom, raztapljanje v 0.2 % sarkozilu.

Nanos 1: hG-CSF standard 0.6 µg

Nanos 2: indukcija pri $OD_{600nm} \approx 6.0$, inkluzijska telesa sprana z vodo

Nanos 3: indukcija pri $OD_{600nm} \approx 6.0$, inkluzijska telesa sprana z 10 mM TrisHCl/pH=8.0 pufrom.

Nanos 4: standardi molekulskih mas (LMW - BioRad)

Nanos 5: indukcija od začetka, inkluzijska telesa sprana z vodo

Nanos 6: indukcija od začetka, inkluzijska telesa sprana z 10 mM TrisHCl/pH=8.0 pufrom.

Slika 2: Primerjava topnosti inkluzijskih teles glede na temperaturo gojenja (25°C ali 37°C).

Metoda: SDS-PAGE (4% zgoščevalni, 15% ločitveni gel, barvano s Coomassie brilliant blue).

Pogoji priprave inkluzijskih teles: temperatura gojenja: 25°C (nanosi 4-10) in 37°C (nanosi 12-18, 20), vodenje fermentacije po principu šaržnih fermentacij, indukcija z 0.4 mM IPTG na začetku, spiranje z ohlajeno vodo ali ohlajenim 10 mM TrisHCl/pH=8.0 pufrom, 1 % Na-deoksiholat, 1 % Triton X-100, 2 M ureo ali 8M ureo.

Nanos 1: standardi molekulskih mas (LMW - BioRad)

Nanos 2: topna frakcija proteinov (supernatant homogenata)

Nanos 3: celotni proteini

Nanos 4: raztopljeni proteini po prvem spiranju inkluzijskih teles z vodo

Nanos 5: raztopljeni proteini po drugem spiranju inkluzijskih teles z vodo

Nanos 6: raztopljeni proteini po prvem spiranju inkluzijskih teles z 10 mM TrisHCl/pH=8.0

Nanos 7: raztopljeni proteini po drugem spiranju inkluzijskih teles z 10 mM TrisHCl/pH=8.0

Nanos 8: raztopljeni proteini po spiranju inkluzijskih teles z 1% Na-deoksiholatom

Nanos 9: raztopljeni proteini po spiranju inkluzijskih teles z 2 M ureo

Nanos 10: raztopljeni proteini po spiranju inkluzijskih teles z 1% Tritonom-X100

Nanos 11: standardi molekulskih mas (LMW - BioRad)

Nanos 12: raztopljeni proteini po prvem spiranju inkluzijskih teles z vodo

Nanos 13: raztopljeni proteini po drugem spiranju inkluzijskih teles z vodo

Nanos 14: raztopljeni proteini po prvem spiranju inkluzijskih teles z 10 mM TrisHCl/pH=8.0

Nanos 15: raztopljeni proteini po drugem spiranju inkluzijskih teles z 10 mM TrisHCl/pH=8.0

Nanos 16: raztopljeni proteini po spiranju inkluzijskih teles z 1% Na-deoksiholatom

Nanos 17: raztopljeni proteini po spiranju inkluzijskih teles z 2 M ureo

Nanos 18: raztopljeni proteini po spiranju inkluzijskih teles z 1% Tritonom-X100

Nanos 19: standard hG-CSF, 0.6 μg

Nanos 20: raztopljeni proteini po spiranju inkluzijskih teles z 8 M ureo

Slika 3: Sestava proteinov v inkluzijskih telesih

Metoda: SDS-PAGE (4% zgoščevalni, 15% ločitveni gel, barvano s Coomassie brilliant blue).

Pogoji priprave inkluzijskih teles: produkcijski sev BL21-SI pET3a/P-Fopt5, temperatura gojenja 25°C, vodenje fermentacije v stresani kulturi, neinducirana kultura ali indukcija z 1.2 M NaCl pri $OD_{600nm} \approx 0.5$ oziroma z začetno indukcijo.

Nanos 1: BL21 (SI) pET3a/P-Fopt5, neinducirana kultura

Nanos 2: BL21 (SI) pET3a/P-Fopt5, kultura inducirana z 1.2 M NaCl pri $OD_{600nm} \approx 0.5$

Nanos 3: BL21 (SI) pET3a/P-Fopt5, kultura inducirana z 1.2 M NaCl pri $OD_{600nm} \approx 0.5$

Nanos 4: BL21 (SI) pET3a/P-Fopt5, kultura inducirana z 1.2 M NaCl od začetka

Nanos 5: BL21 (SI) pET3a/P-Fopt5, kultura inducirana z 1.2 M NaCl od začetka

Nanos 6: rhG-CSF 0.6 μg

Slika 4: Primerjava topnosti inkluzijskih teles glede na spiranje z različnimi topili

Metoda: SDS-PAGE (4% zgoščevalni, 15% ločitveni gel, barvano s Coomassie brilliant blue).

Pogoji priprave inkluzijskih teles: sev BL21(SI) pET3a/P-Fopt5, temperatura gojenja 25^0C, vodenje fermentacije v stresani kulturi, indukcija z 1.2 M NaCl, spiranje z vodo, 10 mM TrisHCl/pH=8.0, 1 % Na-deoksiholat, 1 % Triton X-100 ali 2 M ureo.

Inkluzijska telesa so bila pripravljena v stresani kulturi pri 25^0C, z indukcijo z 1.2 mM NaCl dodanim v medij na začetku.

Nanos 1: topna frakcija proteinov (supernatant homogenata)

Nanos 2: standardi molekulskih mas (LMW - BioRad)
Nanos 3: celotni proteini
Nanos 4: raztopljeni proteini po prvem spiranju inkluzijskih teles z vodo
Nanos 5: raztopljeni proteini po drugem spiranju inkluzijskih teles z vodo
Nanos 6: raztopljeni proteini po prvem spiranju inkluzijskih teles z 10 mM TrisHCl/pH=8.0
Nanos 7: raztopljeni proteini po spiranju inkluzijskih teles z 1% Na-deoksiholatom
Nanos 8: raztopljeni proteini po spiranju inkluzijskih teles z 2 M ureo
Nanos 9: raztopljeni proteini po spiranju inkluzijskih teles z 1% Tritonom-X100

Opis izuma

Presenetljivo smo ugotovili, da lahko pri postopku pridobivanja biološko aktivnega G-CSF, ki je predmet izuma, vodimo biosintezo na tak način, da se izloča pravilno zvit prekurzor G-CSF že v inkluzijskih telesih.

Čeprav so inkluzijska telesa, ki so opisana v patentni in strokovni literaturi, netopni kompaktni agregati nepravilno zvitih in delno pravilno zvitih proteinov, smo pri predloženem izumu dosegli, da se prav v inkluzijskih telesih nahaja že pravilno zvit prekurzor G-CSF. Inkluzijska telesa, ki vsebujejo pravilno zvit prekurzor G-CSF, so bistveno bolj topna od do sedaj znanih težko topnih inkluzijskih teles. Večja topnost inkluzijskih teles omogoča lažje in časovno manj zamudno pridobivanje G-CSF iz inkluzijskih teles. Izkoristki so pri takem načinu pridobivanja G-CSF veliki, kar je uporabno, praktično in ekonomično v industrijskem merilu.

Z izrazom 'pravilno zvit prekurzor' proteina (G-CSF) je mišljen protein (G-CSF), ki ima pravilno konformacijo (trodimenzionalno strukturo), nima pa še vzpostavljenih disulfidnih vezi.

Domnevamo, da se pravilno zvit prekurzor G-CSF nekako ujame v ostale netopne agregate, ki tvorijo inkluzijska telesa, in ga je zato potrebno le izločiti iz teh agregatov. Izločanje poteka s spiranjem in raztapljanjem inkluzijskih teles. Za tako izločanje potrebujemo blaga topila in ne močnih denaturantov, kot je to običajno potrebno v primeru raztapljanja inkluzijskih teles. Ob izločanju pravilno zvitega prekurzorja G-CSF iz inkluzijskih teles poteče spontana oksidacija (prisotnost

zraka...) in s tem spontan nastanek intramolekularnih disulfidnih vezi. Na ta način pridobimo biološko aktivni G-CSF.

Z izrazom 'biološko aktivni G-CSF' je mišljen G-CSF z biološko aktivnostjo, ki je enaka ali večja od $1x10^8$ IU/mg, in se meri z uporabo metode proliferacije na celični liniji, kot je opisano v primeru 12.

V primerjavi z že znanimi postopki pridobivanja heterolognih proteinov, ki se po ekspresiji nahajajo v netopnih inkluzijskih telesih, je postopek, ki je predmet izuma, bolj ekonomičen. Namesto spiranja z detergenti namreč zadostuje že spiranje s pufrom ali vodo, raztapljanje poteka v blagih pogojih namesto v prisotnosti močnih denaturantov in detergentov v visokih koncentracijah. Izognemo se tudi postopkom *in vitro* renaturacije, ki so potrebni za ponovno vzpostavljanje pravilne trodimenzionalne strukture.

Z izrazom 'heterologni protein' je mišljen tisti protein, ki je tuj organizmu, v katerem poteka ekspresija.

Z izrazom 'renaturacija' je mišljena pretvorba denaturiranih proteinov v konformacijo, ki so jo ti proteini imeli pred denaturacijo.

Z izrazom '*in vitro* renaturacija' je mišljena renaturacija, ki je izvedena izven organizma.

Z izrazom 'denaturacija' je mišljen proces, pri katerem se konformacija proteina (trodimenzionalna struktura) spremeni, primarna struktura (aminokislinska veriga, peptidne vezi) proteina pa ostane nespremenjena.

Z izrazom 'denaturant' je mišljena raztopina, v kateri se konformacija proteinov ne ohrani. Biološka aktivnost proteinov se v prisotnosti denaturanta spreminja in se ne ohrani.

Z izrazom 'agregat' je mišljen skupek molekul, ki so med seboj povezane predvsem s hidrofobnimi, pa tudi z drugimi vezmi (kot npr. disulfidnimi). Take molekule niso biološko aktivne.

Postopek pridobivanja biološko aktivnega G-CSF, ki je predmet izuma in omogoča nastanek pravilno zvitega prekurzorja G-CSF že v inkluzijskih telesih in pridobivanje biološko aktivnega G-CSF direktno iz inkluzijskih teles, vključuje način vodenja biosinteze, ki vključuje enega ali kombinacijo več parametrov, ki so izbrani iz skupine, ki sestoji iz:

- temperature gojenja,
- načina indukcije,
- principa vodenja fermentacije,
- sestave gojišča in
- ko-ekspresije pomožnih proteinov.

Z izrazom 'biosinteza' je mišljena produkcija heterolognega proteina s pomočjo mikroorganizmov.

Z izrazom 'gojenje' je mišljena rast mikroorganizmov pod kontroliranimi pogoji submerzno ali na trdnih nosilcih, kjer zagotovimo vir substratov, potrebnih za rast mikroorganizma.

Z izrazom 'fermentacija' je mišljeno gojenje mikroorganizmov v submerznih pogojih v bioreaktorju (fermentorju) ali v stresanih kulturah.

Postopek pridobivanja biološko aktivnega G-CSF, ki je predmet izuma, dodatno vključuje spiranje in raztapljanje inkluzijskih teles ter omogoča izolacijo biološko aktivnega G-CSF brez uporabe denaturantov ali postopka *in vitro* renaturacije.

Bistveno za postopek pridobivanja biološko aktivnega G-CSF, ki je predmet izuma je, da način vodenja biosinteze vključuje parametre, ki omogočajo regulacijo sestave inkluzijskih teles tako, da se poveča delež pravilno zvitih proteinskih molekul G-CSF (prekurzor G-CSF). Visoka akumulacija G-CSF ne pomeni hkrati tudi visokega deleža pravilno zvitega prekurzorja G-CSF. Postopek za pridobivanje biološko aktivnega G-CSF, ki je predmet izuma, omogoča tudi, da se najde optimalno razmerje med akumulacijo G-CSF in deležem pravilno zvitega prekurzorja G-CSF.

To omogoča pridobivanje biološko aktivnega G-CSF z visokimi izkoristki v industrijskem merilu.

Z izrazom 'akumulacija G-CSF' je mišljen delež G-CSF, ki ga dobimo po heterologni ekspresiji gena za G-CSF glede na celotne proteine, ki so prisotni po ekspresiji.

Z izrazom 'heterologna ekspresija' je mišljena ekspresija tistih genov, ki so tuji organizmu, v katerem poteka ekspresija.

Z izrazom 'delež pravilno zvitega prekurzorja G-CSF' je mišljen delež pravilno zvitega prekurzorja G-CSF glede na celotne proteine G-CSF (pravilno, delno pravilno, nepravilno zvite).

Z izrazom 'delež biološko aktivnega G-CSF' je mišljen delež pravilno zvitega prekurzorja G-CSF po spiranju in raztapljanju inkluzijskih teles. Pravilno zvit prekurzor G-CSF po raztapljanju postane biološko aktiven, kajti poteče spontana oksidacija in s tem spontan nastanek disulfidnih vezi.

Postopek pridobivanja biološko aktivnega G-CSF, ki je predmet izuma, omogoča pripravo G-CSF, ki je primeren za klinično uporabo v medicini in veterini.

Uporablja se za pripravo humanega G-CSF in ostalih sesalskih G-CSF in tudi v primeru priprave derivatiziranih oblik G-CSF, kot so: metionil G-CSF (Met-G-CSF), encimsko in kemijsko modificirani (kot npr. pegilirani) G-CSF, G-CSF analogi in fuzijski proteini, ki vsebujejo G-CSF.

Postopek pridobivanja biološko aktivnega G-CSF, ki je predmet izuma, se lahko uporablja tudi v primeru pridobivanja drugih biološko aktivnih heterolognih proteinov, ki se po ekspresiji nahajajo v inkluzijskih telesih. Taki proteini so izbrani iz skupine, ki zajema: interferone (IFN), kot so INF-beta 1b, IFN-beta 2b, IFN-gama 1b, interleukine (IL), kot sta IL-2 in IL-4, granulocitni makrofagni kolonije stimulirajoči dejavnik (GM-CSF), makrofagni kolonije stimulirajoči dejavnik (M-CSF), epidermalni rastni faktor (EGF), humani serumski albumin (HSA), deoksiribonukleazo (DNAza), fibroblastni rastni faktor (FGF), dejavnik tumorske nekroze alfa (TNF alfa) in dejavnik tumorske nekroze beta (TNF-beta). Dodatno lahko zajema vse ostale delno hidrofobne proteine z ne prevelikim številom disulfidnih vezi, ki se v veliki meri spontano zvijejo v pravilno zvito strukturo.

Postopek pridobivanja biološko aktivnega G-CSF, ki je predmet izuma, se uporablja v primeru nastanka inkluzijskih teles po ekspresiji, ne glede na to, kateri organizem se uporablja kot gostitelj za ekspresijo. Lahko se uporabljajo gostiteljski organizmi, ki so izbrani iz skupine, ki zajema predvsem bakterije in kvasovke. Med bakterijskimi sistemi se najbolj pogosto uporabljajo *E. coli* in *Streptomyces* sp., med kvasovkami pa klasične kvasovke, kot je *Saccharomyces cerevisiae*, ter nekovencionalne kvasovke, kot so: *Pichia pastoris, Hansenula polymorpha, Candida utilis* in druge.

Način vodenja biosinteze G-CSF, ki je predmet izuma vključuje parametre, ki so izbrani iz skupine: temperatura gojenja, sestava gojišča, način indukcije, princip vodenja fermentacije in ko-ekspresija pomožnih proteinov. Že z optimizacijo posameznega parametra izmed naštetih parametrov je možno bistveno povečati delež pravilno zvitega prekurzorja G-CSF, še večji delež pa omogoča kombinacija več parametrov.

Temperatura gojenja

Presenetljivo smo ugotovili, da lahko z nižanjem temperature gojenja, omogočimo, da se pravilno zvit prekurzor G-CSF nahaja že v inkluzijskih telesih. Normalna optimalna temperatura gojenja bakterijskih celic je 37°C. V predloženem izumu smo ugotovili, da je prednostna temperatura za pridobivanje pravilno zvitega prekurzorja G-CSF že v inkluzijskih telesih bistveno nižja od 37°C in sicer med 20°C in 30°C. Najbolj prednostna je temperatura okoli 25°C.

Načini indukcije

Presenetljivo smo ugotovili, da je delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih odvisen tudi od načina indukcije. Indukcija je možna z dodatkom induktorja, ki je izbran iz skupine: IPTG, laktoza in NaCl. Prednostna je indukcija z IPTG. Delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih je odvisen tudi od koncentracije induktorja. Pri dodajanju IPTG je koncentracija izbrana v območju od 0.1 mM do 1 mM. Prednostna je koncentracija okoli 0.4 mM. V primeru uporabe NaCl je koncentracija izbrana v območju od 0.3 M do 1.3 M. Prednostna je koncentracija okoli 1.2 M. V primeru uporabe laktoze je koncentracija izbrana v območju med 1 in 10 g/l, najbolj prednostna je koncentracija v območju med okoli 2 in okoli 4 g/l. Delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih je odvisen tudi od načina indukcije (v času gojenja). Induktor lahko dodamo na začetku fermentacije (takoj), kar je prednostno tako v primeru IPTG kot tudi NaCl in laktoze. IPTG in NaCl lahko dodajamo tudi v kasnejših stopnjah fermentacije pri OD_{600nm} okoli 6.0 (OD je merilo za število bakterij).

Princip vodenja fermentacije

Presenetljivo smo ugotovili, da je delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih odvisen tudi od principa vodenja fermentacije. Ta je izbran iz skupine, ki obsega: vodenje fermentacije v bioreaktorju in vodenje fermentacije v

stresani kulturi. Prednostno je vodenje fermentacije v bioreaktorju, ki zajema vodenje fermentacije po principu šaržnih fermentacij in vodenje fermentacije po principu šaržnih fermentacij z dohranjevanjem. Prednosten princip vodenja fermentacije je vodenje fermentacije po principu šaržnih fermentacij, kjer se doseže visoka produktivnost biomase z dobro topnimi inkluzijskimi telesi.

Ko-ekpresija pomožnih proteinov

Ugotovili smo tudi, da je delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih odvisen tudi od dodatkov, ki delujejo stresno. Ti dodatki sprožijo ko-ekspresijo stresnih proteinov in so izbrani iz skupine, ki obsega različne koncentracije etanola in propanola v območju od 1% do 5% (v/v). Najbolj prednostno se uporabljata etanol in propanol v koncentraciji 3% (v/v).

Sestava gojišča

Prav tako smo presenetljivo ugotovili, da je delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih odvisen tudi od sestave gojišča. Gojišča so izbrana iz skupine, ki sestoji iz: GYST/amp100 (20 g/l tripton, 5g/l kvasni ekstrakt, 10 g/l NaCl, 10 g/l glukoze, kovine v sledovih, 100 mg/L ampicilina), GYSP/amp100 (20 g/l fiton, 5g/l kvasni ekstrakt, 10 g/l NaCl, 10 g/l glukoze, kovine v sledovih, 100 mg/L ampicilina) in LYSP/amp100 (20 g/l fiton, 5g/l kvasni ekstrakt, 10 g/l NaCl, 6 g/l glicerola, 2 g/l oziroma 4 g/l laktoze, kovine v sledovih, 100 mg/L ampicilina), LYST/amp100 (20 g/l tripton, 5g/l kvasni ekstrakt, 10 g/l NaCl, 6 g/l glicerola, 2 g/l oziroma 4 g/l laktoze, kovine v sledovih, 100 mg/L ampicilina), LBON/amp100 (10 g/l phyton, 5 g/l yeast extract, 100 mg/ml ampicilina), GYSPON/amp100 (20 g/l phyton, 5g/l kvasni ekstrakt, 10 g/l glukoze, kovine v sledovih, 100 mg/L ampicilina) (Kovine v sledovih: ($FeSO_4x7H_2O$: 40 mg/l, $CaCl_2x2H_2O$: 40 mg/l, $MnSO_4xnH_2O$: 10 mg/l, $AlCl_3x6H_2O$: 10 mg/l, $CoCl_2x6H_2O$: 4 mg/l, $ZnSO_4x7H_2O$: 2 mg/l, $NaMoO_4x2H_2O$: 2 mg/l, $CuSO_4x5H_2O$: 1 mg/l, H_3BO_3: 0,5 mg/l)). Prednostno se uporablja gojišče GYST/amp 100.

Spiranje inkluzijskih teles

Ob povečanju deleža biološko aktivnega proteina v inkluzijskih telesih se bistveno izboljša topnost inkluzijskih teles, kar več ne dovoljuje spiranja z detergenti, ker se preveč pravilno zvitega prekurzorja G-CSF izgubi v izpirku. Spiranje se lahko zato izvede z različnimi pripravki, izbranimi iz skupine: voda in različni pufri v zelo

nizkih koncentracijah (od 1 do 10 mM), kot so npr. pufer Tris/HCl, fosfatni pufer, acetatni pufer, citratni pufer. Najbolj prednostno je spiranje z vodo.

Raztapljanje inkluzijskih teles

Ob povečanju topnosti inkluzijskih teles, ki nastane kot posledica povečanja topnosti pravilno zvitega prekurzorja heterolognega proteina v inkluzijskih telesih, tudi raztapljanje poteka pri blagih pogojih, torej brez dodatka močnih denaturantov ali denaturirajočih koncentracij detergentov. Za raztapljanje inkluzijskih teles se uporabljajo topila izbrana iz skupine, ki sestoji iz uree v nedenaturirajočih koncentracijah (1 - 2 M), N-lauroil sarkozina v nedenaturirajočih koncentracijah (0.05 - 0.25% (m/v)), nizkih koncentracij Zwittergentov, različnih non-detergent sulfobetainov (NDSB), betaina, sarkozina, karbamoil sarkozina, taurina, dimetilsulfoksida (DMSO) in višjih koncentracij nekaterih pufrov, kot so HEPES, HEPPS, MES, ACES, MES. Prednostno se uporabljajo N-lauroil sarkozin, non-detergent sulfobetaini in dimetilsulfoksid (DMSO). Najbolj prednostno se uporablja N-lauroil sarkozin s koncentracijo v območju med 0.1% in 0.25% (m/v).

Temperatura gojenja pri okoli 25°C, vodenje fermentacije po principu šaržnih fermentacij, sestava gojišča GYST/amp100, način indukcije začetna indukcija, indukcija z IPTG s koncentracijo okoli 0.4 mM, spiranje z vodo in raztapljanje inkluzijskih teles v N-lauroil sarkozinu s koncentracijo okoli 0.2% so parametri, pri katerih sta delež pravilno zvitega prekurzorja G-CSF (in s tem delež biološko aktivnega G-CSF) in akumulacija G-CSF visoka. Prav tako je sestava proteinov pri teh parametrih takšna, da lahko na na način pridobljeni G-CSF uporabimo direktno v nadaljnih izolacijskih postopkih, predvsem za direkten nanos na kovinsko kelatno afinitetno kromatografijo - IMAC. Na tak način je možno industrijsko pridobivati G-CSF z visokimi izkoristki.

Primeri

Primer 1: Vpliv temperature gojenja na delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih in na koncentracijo celotnih raztopljenih proteinov po raztapljanju inkluzijskih teles v 0.2% sarkozilu

Humani gen za G-CSF je bil modificiran za visoko ekspresijo v bakteriji *E. coli* (Fopt5). V ekspresijskem sistemu *E. coli* BL21 (DE3) s plazmidom pET3a dosegamo nivo akumulacije, ki lahko predstavlja tudi preko 50 % vseh celičnih proteinov. Kulturo *E. coli* BL21(DE3) pET3a-Fopt5 iz banke sevov –70°C smo nacepili v razmerju 1:100 v LBG/amp medij (10 g/l tripton, 5g/l kvasni ekstrakt, 10 g/l NaCl, 2.5 g/l glukoze, 100 mg/L ampicilina) in gojili 10 ur na stresalniku 25°C, 160 rpm. 2 do 4 ml tako pripravljene kulture smo precepili v 40 ml GYST/amp100 medija, kateremu smo že na začetku dodali induktor IPTG do končne koncentracije 0.4 mM, (začetni $OD_{\lambda 600nm}$ kulture je znašal 0.385). Kulturo smo nato gojili na stresalniku (stresana kultura) pri treh različnih temperaturah in 160 rpm do eksponencialne faze rasti:

- pri 25°C: 22 ur, končni $OD_{\lambda 600nm}$ kulture je znašal 15.2 ± 0.5;
- pri 37°C: 15 ur, končni $OD_{\lambda 600nm}$ kulture je znašal 12.2 ± 0.4;
- pri 42°C: 15 ur, končni $OD_{\lambda 600nm}$ kulture je znašal 6.75 ± 0.2.

Tako pripravljene kulture smo po koncu gojenja centrifugirali 10 minut na 5000 rpm, sprali z 4-5 kratnim volumnom 10 mM TrisHCl/pH=8.0 ter biomaso prenesli v nove centrifugirke ter ponovno centrifugirali 10 minut na 5000 rpm. Biomaso smo resuspendirali v 5 kratnem volumnu 10 mM TrisHCl/pH=8.0 pufra in sonicirali 12 krat po 1 minuto s potopno sondo (duty cycle: 60%, moč: 7, pulzi: 2 s^{-1}). Po soniciranju smo razbite celice ponovno centrifugirali 30 minut na 5000 rpm. Pelet z inkluzijskimi telesi smo raztopili pri nedenaturirajočih pogojih v 50 kratnem volumnu 0,2 % N-lauroil sarkozina v 40 mM TrisHCl/pH=8.0, raztapljanje je potekalo prekonočno 16 do 18 ur na sobni temperaturi pri stresanju 50 rpm. Po raztapljanju smo zmerili koncentracijo proteinov po Bradfordu na standard čistega hMet-G-CSF. Koncentracije so znašale okrog 1 mg/ml za 42°C in 37°C ter med 2 in 3 mg/ml za 25°C. Koncentracija celotnih proteinov po raztapljanju inkluzijskih teles v 0.2% N-lauroil sarkozina in delež pravilno zvitega prekurzorja G-CSF oz biološko aktivnega G-CSF, pripravljenega iz biomase gojene pri različnih temperaturah je prikazan v tabeli 1.

Delež pravilno zvitega zvitega prekurzorja G-CSF oz. biološko aktivnega G-CSF (po izločitvi iz inkluzijskih teles) je bil določen z merjenjem biološke aktivnosti raztopljenih inkluzijskih teles brez odstranitve N-lauroil sarkozina (pri taki diluciji vzorcev sarkozil ne moti).

Temperatura gojenja	Koncentracija celotnih proteinov po raztapljanju inkluzijskih teles v 0,2 % N-lauroil sarkozinu	Delež biološko aktivnega G-CSF
25°C	2.7 mg/ml	> 30 %
37°C	1.1 mg/ml	> 20 %
42°C	1.0 mg/ml	< 1 %

Tabela 1: Koncentracija celotnih proteinov po raztapljanju inkluzijskih teles v 0,2 % N-lauroil sarkozina in delež pravilno zvitega prekurzorja G-CSF oz. biološko aktivnega G-CSF, pripravljenega iz biomase gojene pri različnih temperaturah.

Iz Tabele 1 je razvidno, da smo delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih z gojenjem pri znižani temperaturi bistveno povečali. Prav tako smo pri znižani temperaturi dobili do 2.5 krat višje koncentracije celotnih proteinov po raztapljanju inkluzijskih teles v 0,2 % N-lauroil sarkozina, kar pomeni, da so tako pripravljena inkluzijska telesa bistveno bolj topna.

Primer 2: Vpliv načina indukcije IPTG na akumulacijo G-CSF

Indukcija z 0.4 mM IPTG na začetku fermentacije

Predhodni poskusi z različnimi koncentracijami IPTG (0.05 mM - 0.4 mM) so pokazali, da je dovolj visoka in skoraj enaka akumulacija G-CSF dosežena v področju 0.1 - 0.4 mM za biomase s končnim $OD_{\lambda 600nm}$ = 20-30.

Kulturo *E. coli* BL21(DE3) pET3a-Fopt5 iz banke sevov –70°C smo nacepili v razmerju 1:500 v LBG/amp medij in gojili 14-18 ur na stresalniku 25°C, 150 rpm. Tako pripravljeno kulturo smo uporabili kot vcepek za fermentor v razmerju 1:20 v produkcijskem mediju GYST/amp, kateremu smo že na začetku dodali induktor IPTG do končne koncentracije 0.4 mM. Fermentacijo smo vodili po principu šaržnih

fermentacij 20-23 ur pri 25°C, 500 rpm in pretoku zraka 1 vvm (7 L Chemap fermentor). Po koncu procesa je $OD_{\lambda 600nm}$ kulture znašal približno 25.

Biomaso smo centrifugirali 5 minut na 5000 rpm (Beckman centrifuga) in zamrznili za nadaljno obdelavo. Po SDS-PAGE in barvanju s Coomassie barvilom smo s profilno denzitometrično analizo določili delež G-CSF v celokupnih proteinih, ki je znašal med 30% in 40%.

Indukcija z 0.4 mM IPTG pri $OD_{600nm} \approx 6.0$

Fermentacijo smo izvedli na podoben način kot pri indukciji z 0.4 mM IPTG od začetka, le da smo induktor IPTG do končne koncentracije 0.4 mM v medij dodali šele, ko je kultura dosegla $OD_{600nm} \approx 6.0$. Fermentacijo smo vodili po principu šaržnih fermentacij 18-20 ur pri 25°C, 500 rpm in pretoku zraka 1 vvm (7 L Chemap fermentor). Po koncu procesa je $OD_{\lambda 600nm}$ kulture znašal približno 30. Biomaso smo centrifugirali 5 minut na 5000 rpm (Beckman centrifuga) in zamrznili za nadaljno obdelavo. Po SDS-PAGE in barvanju s Coomassie barvilom smo s profilno denzitometrično analizo določili delež G-CSF v celokupnih proteinih, ki je znašal med 30% in 40%. Iz biomase smo izolirali inkluzijska telesa, kot je opisano v Primeru 3. Pelet z inkluzijskimi telesi smo sprali z ohlajeno vodo ali ohlajenim 10 mM TrisHCl/pH=8 pufrom, ponovno centrifugirali 30 minut na 10000 rpm in raztopili pri nedenaturirajočih pogojih v 50 kratnem volumnu 0,2 % N-lauroil sarkozina v 40 mM TrisHCl/pH=8.0. Raztapljanje je potekalo prekonočno 16 do 18 ur na sobni temperaturi pri stresanju 100 - 150 rpm. Po raztapljanju smo zmerili koncentracijo proteinov po Bradfordu na standard čistega hMet-G-CSF.

Koncentracija celotnih raztopljenih proteinov po obeh načinih indukcije je bila med 2 in 4 mg/ml.

Primerjava deleža hG-CSF v inkluzijskih telesih, ko dodamo IPTG na začetku fermentacije (indukcija takoj) ali ko OD_{600nm} kulture znaša okoli 6.0

Ko dodamo IPTG v medij že na začetku, dobimo inkluzijska telesa z zelo visoko vsebnostjo G-CSF in zelo malo primesi drugih *E. coli* proteinov. V primeru indukcije pri $OD_{600nm} \approx 6.0$, je vsebnost G-CSF v inkluzijskih telesih sicer še vedno visoka, vendar je vsebnost drugih *E. coli* proteinov višja in lahko moti v nadaljnjih stopnjah izolacije. (Slika 1)

Primer 3: Vpliv temperature gojenja na topnost inkluzijskih teles

Topnost inkluzijskih teles se po gojenju celic pri 25°C bistveno izboljša. Zato spiranje inkluzijskih teles ni dovoljeno z detergenti, ker se preveč pravilno zvitega prekurzorja G-CSF izgubi v izpirku. Vendar je spiranje, vsaj z vodo, nujno zaradi odstranitve nekaterih proteinov, ki bi sicer lahko motili pri kromatografiji.

Priprava biomase: Kulturo *E. coli* BL21(DE3) pET3a/P-Fopt5 iz banke sevov –70°C smo nacepili v razmerju 1:500 v LBG/amp medij in gojili 14-18 ur na stresalniku 25°C, 150 rpm. Tako pripravljeno kulturo smo uporabili kot vcepek za fermentor v razmerju 1:20 v produkcijskem mediju GYST/amp, kateremu smo že na začetku dodali induktor IPTG do končne koncentracije 0.4 mM. Fermentacijo smo vodili po principu šaržnih fermentacij pri 500 rpm in pretoku zraka 1 vvm (7 L Chemap fermentor) pri dveh različnih temperaturah:

pri 25°C 18-25 ur, po koncu procesa je $OD_{\lambda 600nm}$ kulture znašal približno 25.

pri 37°C 8.25 ur, po koncu procesa je $OD_{\lambda 600nm}$ kulture znašal približno 28.

Izolacija inkluzijskih teles: Po končani fermentaciji smo bakterijski pelet ločili od gojišča s centrifugiranjem pri +4°C in 5000 rpm. Mokri bakterijski pelet smo resuspendirali v 4-kratnim volumnu pufra X (10 mM Tris/HCl, pH=8,0). Homogenizacijo vzorca smo izvedli z ultraturaksom, nato pa smo bakterijske celice razbili s homogenizatorjem EmulsiFlex C-5 firme AVESTIN v eni pasaži pri razliki tlakov med 10000 – 15000 psi (70 – 110 MPa). Po 30-minutnem centrifugiranju pri 10000 rpm smo supernatant, ki vsebuje topne proteine bakterije *E. coli*, zavrgli, pelet (inkluzijska telesa) pa smo zamrznili na –20°C in ga uporabili za poskuse spiranja in raztapljanja inkluzijskih teles.

Izvedeni so bili naslednji poskusi spiranja inkluzijskih teles (Slika 2):

A. Spiranje inkluzijskih teles z vodo

Zatehtano količino inkluzijskih teles smo resuspendirali v 10-kratni količini ohlajene vode (+4°C), centrifugirali 10 minut pri 10000 rpm na +4°C in postopek ponovili z enako količino ohlajene vode. V supernatantih po obeh spiranjih smo določili količino proteinov po Bradfordu (glede na goveji serumski albumin (BSA) kot standard) in analizirali proteinsko sestavo s SDS-PAGE.

Kvaliteta vode: voda je pripravljena z aparaturo Milli-Q RG (Millipore).

B. Spiranje inkluzijskih teles s pufrom Y (10mM Tris/HCl, pH 8,0)

Zatehtano količino inkluzijskih teles smo resuspendirali v 10-kratni količini ohlajenega pufra Y (10 mM Tris/HCl, pH 8,0) (+4°C), centrifugirali 10 minut pri 10000 rpm na +4°C in postopek ponovili z enako količino ohlajene vode. V supernatantih po obeh spiranjih smo določili količino proteinov po Bradfordu (glede na BSA kot standard) in analizirali proteinsko sestavo s SDS-PAGE.

C. Spiranje inkluzijskih teles z 1 % Na-deoksiholatom

Zatehtano količino inkluzijskih teles smo resuspendirali v 10-kratni količini ohlajenega (+4°C) pufra W (50 mM Tris/HCl, pH 9,0 z dodatkom 1% Na deoksiholata, 5 mM ditiotreitola (DTT) in 5 mM EDTA, pustili 30 minut na ledu in centrifugirali 10 minut pri 10000 rpm na +4°C. V supernatantih smo določili količino proteinov po Bradfordu (glede na BSA kot standard) in analizirali proteinsko sestavo s SDS-PAGE.

D. Spiranje inkluzijskih teles z 1 % Triton X-100

Zatehtano količino inkluzijskih teles smo resuspendirali v 10-kratni količini ohlajenega (+4°C) pufra X (10 mM Tris/HCl, pH=8,0) z dodatkom 1 % Triton X-100, pustili 30 minut na ledu in centrifugirali 10 minut pri 10000 rpm na +4°C. V supernatantih smo določili količino proteinov po Bradfordu (glede na BSA kot standard) in analizirali proteinsko sestavo s SDS-PAGE.

E. Spiranje inkluzijskih teles z 2 M ureo

Zatehtano količino inkluzijskih teles smo resuspendirali v 10-kratni količini ohlajenega (+4°C) pufra X (10 mM Tris/HCl, pH=8,0) z dodatkom 2M uree, pustili 30 minut na ledu in centrifugirali 10 minut pri 10000 rpm na +4°C. V supernatantih smo določili količino proteinov po Bradfordu (glede na BSA kot standard) in analizirali proteinsko sestavo s SDS-PAGE.

F. Spiranje z 8 M ureo

Zatehtano količino inkluzijskih teles smo resuspendirali v 10-kratni količini ohlajenega (+4°C) pufra X (10 mM Tris/HCl, pH=8,0) z dodatkom 8 M uree, pustili 30 minut na ledu in centrifugirali 10 minut pri 10000 rpm na +4°C. V supernatantih smo določili količino proteinov po Bradfordu (glede na BSA kot standard) in analizirali proteinsko sestavo s SDS-PAGE.

Iz slike 2 je razvidno, da so inkluzijska telesa, ki smo jih dobili pri temperaturi gojenja 25°C (nanosi 4-10) bistveno bolj topna, kot so inkluzijska telesa, ki smo jih

dobili pri temperaturi gojenja 37°C (nanosi 12-18, 20). V primeru gojenja pri 25°C spiranje z detergenti (1% deoksiholat, 1% Triton-X100) in z 2 M ureo ni več mogoče, kajti večino heterolognega proteina se izgubi v izpirku (nanosi 8, 9, 10), v primeru gojenja pri 37°C pa je spiranje z detergenti (1% deoksiholat, nanos 16), 2 M ureo (nanos 17) in 1% Tritonom-X100 (nanos 18) še vedno mogoče. Vidimo tudi, da je inkluzijska telesa, ki smo jih dobili pri temperaturi gojenja 37°C možno raztopiti šele z 8 M ureo (nanos 20).

Primer 4: Vpliv principa vodenja fermentacije na akumulacijo G-CSF in na delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih

Visok delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih lahko dobimo z vodenjem po principu šaržnih fermentacij in po principu šaržnih fermentacij z dohranjevanjem

Vodenje fermentacije po principu šaržnih fermentacij

Kulturo *E. coli* BL21(DE3) pET3a-Fopt5 iz banke sevov –70°C smo nacepili v razmerju 1:500 v LBG/amp medij in gojili 14-18 ur na stresalniku 25°C, 150 rpm. Tako pripravljeno kulturo smo uporabili kot vcepek za fermentor v razmerju 1:20 v produkcijskem mediju GYST/amp, kateremu smo že na začetku ali v zgodnji eksponencialni fazi, ko je $OD_{\lambda 600nm}$ kulture znašal 6, dodali induktor IPTG do končne koncentracije 0.4 mM. Fermentacijo smo vodili po principu šaržnih fermentacij 18-25 ur pri 25°C, 500 rpm in pretoku zraka 1 vvm (7 L Chemap fermentor). Po koncu procesa je $OD_{\lambda 600nm}$ kulture znašal približno 25 v primeru začetne indukcije in 30 v primeru kasnejše indukcije. Biomaso smo centrifugirali 5 minut na 5000 rpm (Beckman centrifuga) in zamrznili za nadaljno obdelavo. Po SDS-PAGE in barvanju s Coomassie barvilom smo s profilno denzitometrično analizo določili akumulacijo G-CSF, ki je znašala med 30% in 40%.

Vodenje fermentacije po principu šaržnih fermentacij z dohranjevanjem

Fermentacijo smo vodili po principu šaržnih fermentacij z dohranjevanjem 25.5-30 ur pri 25°C, 500 rpm in pretoku zraka 1 vvm (7 L Chemap fermentor). Po izrabi glukoze v mediju, t.j. po koncu šaržnega procesa, (vodenega kot opisano prej), ko je pH vrednost medija narasla, smo začeli z dohranjevanjem z raztopino 20 % glukoze z 100 mg/l ampicilina, da je bila specifična hitrost rasti μ med 0.05 in 0.1 hr^{-1}.

Proces smo zaključili po 7-7.5 urah dohranjevanja (skupaj 25.5-30 ur), ko je $OD_{\lambda 600nm}$ kulture znašal približno 42. Biomaso smo centrifugirali 5 minut na 5000 rpm (Beckman centrifuga) in zamrznili za nadaljno obdelavo. Po SDS-PAGE in barvanju s Coomassie barvilom smo s profilno denzitometrično analizo akumulacijo G-CSF, ki se ohranja na podobno visokem nivoju kot v primeru vodenja po principu šaržnih fermentacij, to je med 30% in 40%. Iz biomase smo izolirali inkluzijska telesa, kot je opisano v Primeru 3. Pelet z inkluzijskimi telesi smo sprali z ohlajeno vodo, ponovno centrifugirali 30 minut na 10000 rpm in raztopili pri nedenaturirajočih pogojih v 50 kratnem volumnu 0,2 % N-lauroil sarkozina v 40 mM TrisHCl/pH=8.0. Raztapljanje je potekalo prekonočno 16 do 18 ur na sobni temperaturi pri stresanju 100 - 150 rpm. Po raztapljanju smo zmerili koncentracijo proteinov po Bradfordu na standard čistega hMet-G-CSF.

Koncentracije proteinov pri obeh principih vodenja fermentacije so znašale med 2 in 4 mg/ml. Delež pravilno zvitega prekurzorja G-CSF oz delež biološko aktivnega G-CSF je bil določen z merjenjem biološke aktivnost raztopljenih inkluzijskih teles brez odstranitve N-lauroil sarkozina (pri taki diluciji vzorcev sarkozil ne moti).

Princip vodenja fermentacije	Način indukcije z 0.4 mM IPTG	Delež biološko aktivnega G-CSF
šaržni	začetna	44 %
šaržni z dohranjevanjem	začetna	39 %
šaržni	$OD_{600nm} \approx 6$	43 %
šaržni z dohranjevanjem	$OD_{600nm} \approx 6$	37 %

Tabela 2: Delež pravilno zvitega prekurzorja G-CSF glede na način vodenja fermentacije in glede na način indukcije.

Iz tabele 2 je razvidno, da dobimo v vseh primerih visok delež biološko aktivnega G-CSF, delež je največji v primeru, ko vodimo fermentacijo po principu šaržnih fermentacij.

Primer 5: Vpliv indukcije z laktozo na akumulacijo G-CSF in na delež pravilno zvitega prekurzorja G-CSF

Kulturo *E. coli* BL21(DE3) pET3a-Fopt5 iz banke sevov –70°C smo nacepili v razmerju 1:500 v LBG/amp medij in gojili 14-18 ur na stresalniku 25°C, 150 rpm. Tako pripravljeno kulturo smo uporabili kot vcepek za fermentor v razmerju 1:20 v modificiranem produkcijskem mediju LYST/amp, kjer smo kot vir ogljika namesto glukoze uporabili glicerol (6 g/l) in laktozo (2 g/l oziroma 4 g/l), ki je bila hkrati tudi induktor ekspresije namesto IPTG. Fermentacijo smo vodili po principu šaržnih fermentacij 17-21 ur pri 25°C, 500 rpm in pretoku zraka 1 vvm (7 L Chemap fermentor). Po koncu procesa je $OD_{\lambda 600nm}$ kulture znašal približno 20. Biomaso smo centrifugirali 5 minut na 5000 rpm (Beckman centrifuga), 1 krat sprali z 10 mM Tris/HCl, pH=8 pufrom in zamrznili za nadaljno obdelavo. Po SDS-PAGE in barvanju s Coomassie barvilom smo s profilno denzitometrično analizo določili akumulacijo G-CSF, ki znaša 27 % pri indukciji z 2 g/l laktoze oziroma 33 % pri indukciji s 4 g/l laktoze. Iz biomase smo izolirali inkluzijska telesa, kot je opisano v Primeru 3. Pelet z inkluzijskimi telesi smo sprali z ohlajeno vodo, ponovno centrifugirali 30 minut na 10000 rpm in raztopili pri nedenaturirajočih pogojih v 50 kratnem volumnu 0.2 % N-lauroil sarkozina v 40 mM TrisHCl/pH=8.0. Raztapljanje je potekalo prekonočno 16 do 18 ur na sobni temperaturi pri stresanju 100 - 150 rpm.

Ekspresijski sistem	Indukcija z laktozo	Temperatura gojenja	Delež biološko aktivnega G-CSF
pET3a / P-Fopt5 *E. coli* BL21 (DE3)	2 g/l	25°C	≈ 25 %
pET3a / P-Fopt5 *E. coli* BL21 (DE3)	4 g/l	25°C	≈ 23 %

Tabela 3: Delež pravilno zvitega prekurzorja G-CSF oz. deleži biološko aktivnega G-CSF (v %) v inkluzijskih telesih, ki jih dobimo z indukcijo produkcijskega seva *E. coli* BL21 (DE3) pET3a/P-Fopt5 z laktozo.

Iz Tabele 3 je razvidno, da smo tudi z indukcijo z laktozo uspeli dobiti več kot 23 % delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih z gojenjem pri temperaturi 25^{0}C.

Primer 6: Vpliv indukcije z NaCl na akumulacijo G-CSF in na delež pravilno zvitega prekurzorja G-CSF

Kulturo *E. coli* BL21-SI pET3a/P-Fopt5 iz banke sevov –70^{0}C smo nacepili v razmerju 1:20 v LBON/amp medij in gojili prekodnevno 8 ur na stresalniku 25^{0}C, 160 rpm. 1 ml tako pripravljene kulture smo precepili v 20 ml GYSPON/amp100 medija, kateremu smo dodali induktor NaCl do končne koncentracije 1.2 M že na začetku ali pri $OD_{\lambda 600nm} \approx 0.5$, to je po približno 3 urah gojenja. Kulturo smo v obeh primerih gojili na stresalniku pri temperaturi 25^{0}C in 160 rpm 20-24 ur. Za analizo na SDS-PAGE smo 5 ml tako pripravljene kulture po koncu gojenja centrifugirali 5 minut na 5000 rpm. Pelete smo nato resuspendirali v 15 ml 10 mM TrisHCl/pH=8.0. Vzorce smo zmešali v razmerju 3:1 s 4x SDS - vzorčnim pufrom z DTT (pH=8.7) in segrevali 10 minut na 95^{0}C, odcentrifugirali ter tako pripravljene nanesli na gel. Akumulacija G-CSF je navedena v tabeli 4, kjer vidimo, da dobimo višjo akumulacijo G-CSF, ko dodamo 1.2 M NaCl v medij na začetku, to je ob inokulaciji in ne standardno pri $OD_{\lambda 600nm} \approx 0.5$, kot priporoča proizvajalec (Life Technologies).

Ekspresijski sistem	Pogoji gojenja in indukcije	Temperatura gojenja	Akumulacija G-CSF
pET3a / P-Fopt5 *E. coli* BL21-SI	GYSPON/amp100 1.2 M NaCl na začetku	25° C	25 %
pET3a / P-Fopt5 *E. coli* BL21-SI	GYSPON/amp100 1.2 M NaCl pri $OD_{\lambda 600nm} \approx 0.5$,	25° C	17 %

Tabela 4: Primerjava akumulacije G-CSF produkcijskega seva BL21-SI pET3a/P-Fopt5 pri različnih gojiščih in načinih indukcije.

Navedene vrednosti za vsebnost G-CSF so bile dobljene z denzitometrično analizo SDS-PAGE gelov obarvanih s Coomassie brilliant blue (Slika 3). Relativni

delež je bil določen s profilno analizo (program Molecular analyst; BioRad) gelov na aparatu Imaging densitometer Model GS670 (BioRad).

Priprava vzorcev za določanje biološke aktivnosti in poskuse spiranja inkluzijskih teles

Za analizo biološke aktivnosti ter poskuse spiranja inkluzijskih teles smo pripravili večjo količino biomase. Kulturo *E. coli* BL21-SI pET3a-Fopt5 iz banke sevov –70°C smo nacepili v razmerju 1:20 v LBON/amp medij in gojili prekodnevno 8 ur na stresalniku 25°C, 160 rpm. Po 10 ml tako pripravljene kulture smo precepili v 8 x 200 ml GYSPON/amp100 medija, kateremu smo dodali induktor NaCl do končne koncentracije 1.2 mM že na začetku. Kulturo smo gojili na stresalniku pri temperaturi 25°C in 160 rpm 24 ur. Iz biomase smo izolirali inkluzijska telesa, kot je opisano v Primeru 3. Pelet z inkluzijskimi telesi smo sprali z ohlajeno vodo in jih raztopili z dodatkom 0,2 % N-lauroil sarkozina kot je opisano v Primeru 9. Po raztapljanju smo zmerili koncentracijo proteinov po Bradfordu na standard čistega hMet-G-CSF. Koncentracija celotnih raztopljenih proteinov je bila med 2 in 3 mg/ml.

Ekspresijski sistem	Temperatura gojenja	Delež biološko aktivnega G-CSF
pET3a / P-Fopt5 *E. coli* BL21-SI	25°C	≈ 40 %

Tabela 5: Delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih oz delež biološko aktivnega G-CSF, ki ga dobimo z indukcijo produkcijskega seva *E. coli* BL21-SI pET3a / P-Fopt5 z 1.2 M NaCl.

Iz Tabele 5 je razvidno, da smo dobili tudi s produkcijskim sevom *E. coli* BL21-SI pET3a/P-Fopt5, kjer smo inducirali ekspresijo heterolognega gena z 1.2 M NaCl, visok delež pravilno zvitega prekurzorja G-CSF (oz. biološko aktivnega G-CSF) v inkluzijskih telesih z gojenjem pri temperaturi 25°C.

Topnost inkluzijskih teles

Tudi s produkcijskim sevom *E. coli* BL21-SI pET3a/P-Fopt5, kjer smo inducirali ekspresijo heterolognega gena z 1.2 M NaCl, smo dobili z gojenjem pri temperaturi 25°C bolj topna inkluzijska telesa. Tako tudi v tem primeru ni dovoljeno spiranje inkluzijskih teles z detergenti, ker se preveč pravilno zvitega prekurzorja G-CSF izgubi v izpirku (Slika 4).

Primer 7: Vpliv dodatkov, ki delujejo stresno, npr. etanola ali propanola, na delež pravilno zvitega prekurzorja G-CSF v inkluzijskih telesih

Kulturo *E. coli* BL21(DE3) pET3a-Fopt5 iz banke sevov –70°C smo nacepili v razmerju 1:500 v LBPG/amp100 medij (10 g/l phyton, 5 g/l yeast extract, 10 g/l NaCl, 100 mg/ml ampicilina) in gojili 17 ur na stresalniku 25°C, 160 rpm. 10 ml tako pripravljene kulture smo precepili v 200 ml medija s takojšnjim dodatkom induktorja IPTG do končne koncentracije 0.4 mM:

- GYSP/amp100 medija (kontrola);
- GYSP/amp100 medija z dodatkom etanola (končna koncentracija etanola v mediju 3%);
- GYSP/amp100 medija z dodatkom izo-propanola (končna koncentracija izo-propanola v mediju 3%).

Kulturo smo nato gojili na stresalniku pri temperaturi 25°C in 160 rpm 24 ur v primeru kontrole GYSP/amp100 medija. V primeru GYSP/amp100 medija z dodatkom etanola ali izo-propanola smo kulture gojili pod enakimi pogoji 34 ur, saj dodatek enega ali drugega upočasni rast. Iz biomase smo izolirali inkluzijska telesa, kot je opisano v Primeru 3. Pelet z inkluzijskimi telesi smo sprali z ohlajeno vodo in jih raztopili z dodatkom 0,2 % N-lauroil sarkozina kot je opisano v Primeru 9.

Ekspresijski sistem	Temperatura gojenja v ^{0}C	Dodatek	Delež biološko aktivnega G-CSF
pET3a / P-Fopt5 *E. coli* BL21 (DE3)	25^{0}C	brez dodatka	≈ 50 %
pET3a / P-Fopt5 *E. coli* BL21 (DE3)	25^{0}C	3 % etanol	≈ 59 %
pET3a / P-Fopt5 *E. coli* BL21 (DE3)	25^{0}C	3 % propanol	≈ 62 %

Tabela 6: Delež pravilno zvitega prekurzorja G-CSF (v %) v inkluzijskih telesih, ki jih dobimo z indukcijo produkcijskega seva *E. coli* BL21 (DE3) pET3a / P-Fopt5 v GYSP/amp100 mediju z 0.4 mM IPTG in z dodatki etanola ali propanola, ki delujeta stresno.

Iz Tabele 6 je razvidno, da dodatki etanola ali propanola, ki sprožita stresne proteine, povečajo delež pravilno zvitega prekurzorja G-CSF oz. delež biološko aktivnega G-CSF v inkluzijskih telesih.

Primer 8: Raztapljanje inkluzijskih teles z 2 M ureo v pufru Z (40mM Tris/HCl, pH 8,0)

K 0,30 g s hladno vodo spranih inkluzijskih teles (pripravljenih, kot je opisano v Primeru 3) smo dodali 12 ml pufra Z (40 mM Tris/HCl pH=8.0) z 2 M ureo. Po homogenizaciji smo raztapljali med rahlim stresanjem pri 80 rpm pri 20^{0}C 16 ur. Nato smo centrifugirali 10 minut pri 14000 rpm in 10 ^{0}C in pelet zavrgli. Supernatant, ki vsebuje raztopljene proteine inkluzijskih teles, pa smo uporabili za določitev celokupnih proteinov, SDS-PAGE analizo in določitev biološke aktivnosti.

Koncentracija proteinov po Bradfordu (glede na hMetG-CSF): 2,6 mg/ml, celokupni raztopljeni proteini: 31 mg. (Količina raztopljenih proteinov: približno 10 % mase inkluzijskih teles).

Biološka aktivnost G-CSF: okrog 1,5x10^7 IU/mg

Primer 9: Raztapljanje inkluzijskih teles z 0,2 % N-lauroil-sarkozin v pufru Z (40 mM Tris/HCl, pH 8,0)

Na 0,31 g s hladno vodo spranih inkluzijskih teles (pripravljenih, kot je opisano v Primeru 3) smo dodali 15,6 ml pufra Z (40 mM Tris/HCl pH=8.0) z 0.2% N-lauroil-sarkozina. Po homogenizaciji smo raztapljali 1 uro pri sobni temperaturi (20-22^{0}C) med stresanjem na 100-150 rpm. Za pospešitev oksidacije oziroma tvorbe disulfidnih vezi smo nato dodali 0,1 M $CuSO_4 \times 5H_2O$ do končne koncentracije 40 µM. Stresanje smo nadaljevali preko noči (16 ur) na stresalniku pri 80 rpm in temperaturi 20^{0}C. Naslednji dan smo centrifugiramo 10 minut pri 14000 rpm in +10 ^{0}C in pelet zavrgli. V supernatantu z raztopljenimi proteini inkluzijskih teles smo odstranili N-lauroil-sarkozin z DOWEX-om (DOWEX 1 Sigma), tako da smo dodali 0,39 g DOWEX 1, stresali 1 uro na sobni temperaturi (20-22^{0}C) in 100-150 rpm, nato pa proteinsko raztopino oddekantirali. Tako pripravljeno raztopino inkluzijskih teles smo uporabili za določitev celokupnih proteinov, SDS-PAGE analizo in za določitev biološke aktivnosti. Koncentracija proteinov po Bradfordu (glede na hMetG-CSF) 4,4 mg/ml, celokupni raztopljeni proteini: 68 mg, (količina raztopljenih proteinov: približno 20 % mase inkluzijskih teles).

Biološka aktivnost G-CSF: okrog 4,0 - 4,5 x10^7 IU/mg – ni bistvenih razlik, če je dodan $CuSO_4 \times 5H_2O$ ali ne.

Primer 10: Raztapljanje inkluzijskih teles z 0,2 % NDSB (195, 201, 211, 256) v pufru Z (40 mM Tris/HCl, pH 8,0)

Na približno 0,16 g alikvote s hladno vodo spranih inkluzijskih teles (pripravljenih, kot je opisano v Primeru 3) smo dodali 40-kratni prebitek pufra Z (40 mM Tris/HCl pH=8.0) z dodatkom različnih NDSB (non-detergent sulfobetaine) v koncentraciji 0, 2%. Uporabljeni so bili NDSB 195, NDSB 201, NDSB 211 in NDSB 256. Po homogenizaciji smo vzorce inkluzijskih teles raztapljali pri temperaturi 20 ^{0}C med stresanjem pri 80 rpm preko noči. Vzporedno smo raztapljali tudi vzorce inkluzijskih teles, pri katerih smo po začetnem 30-minutnem raztapljanju za pospečitev oksidacije dodali raztopino 0,1 M $CuSO_4 \times 5H_2O$ do končne koncentracije 40 µM in nato nadaljevali stresanje preko noči. Naslednjega dne smo po centrifugiranju oborine zavrgli, supernatante pa brez vsake predhodne obdelave

uporabili za določitev celokupnih proteinov, SDS-PAGE analizo in za določitev biološke aktivnosti. Koncentracija proteinov po Bradfordu (glede na hMetG-CSF): v vseh primerih okrog 3,6 mg/ml, torej skupno okrog 24 mg celokupnih proteinov (Količina raztopljenih proteinov: približno 15 % mase inkluzijskih teles).

Biološka aktivnost G-CSF: okrog 3-4x10^7 IU/mg – ni bistvenih razlik, če je dodan $CuSO_4 \times 5H_2O$ ali ne.

Primer 11: Raztapljanje inkluzijskih teles s 5 % DMSO v pufru Z (40mM Tris/HCl, pH 8,0)

Na 0,16 g s hladno vodo spranih inkluzijskih teles (pripravljenih, kot je opisano v Primeru 3) smo dodali 40-kratni prebitek pufra Z (40 mM Tris/HCl pH=8.0) z dodatkom 5 % DMSO. Po homogenizaciji smo inkluzijska telesa raztapljali pri temperaturi 20 ^{0}C med stresanjem pri 80 rpm preko noči. Vzporedno smo raztapljali tudi vzorec inkluzijskih teles, pri katerih smo po začetnem 30-minutnem raztapljanju za pospečitev oksidacije dodali raztopino 0,1 M $CuSO_4$ x $5H_2O$ do končne koncentracije 40 μM in nato nadaljevali stresanje preko noči. Naslednjega dne smo po centrifugiranju oborine zavrgli, supernatante pa brez vsake predhodne obdelave uporabili za določitev celokupnih proteinov, SDS-PAGE analizo in za določitev biološke aktivnosti. Koncentracija proteinov po Bradfordu (glede na hMetG-CSF): okrog 3,6 mg/ml, torej skupno okrog 24 mg celokupnih proteinov (Količina raztopljenih proteinov: približno 15 % mase inkluzijskih teles).

Biološka aktivnost G-CSF: okrog 4x10^7 IU/mg – ni bistvenih razlik, če je dodan $CuSO_4 \times 5H_2O$ ali ne.

Primer 12: Testiranje biološke aktivnosti G-CSF *in vitro*

Biološko aktivnost G-CSF smo določali z metodo proliferacije na celični liniji NFS-60 po znani metodi (Hammerlingn, U. s sodelavci v *J Pharm Biomed Anal* **13**, 9-20 (1995)) in uporabi mednarodnega standarda Human recombinant G-CSF (88/502, yeast cell derived; NIBSC Potters Bar, Hertfordshire, UK); (Mire-Sluis,A.R. s sodelavci v *J Immunol Methods* **179**, 117-126 (1995)).

Lek farmacevtska družba d. d.

Patentni zahtevki

1. Postopek pridobivanja proteinov, označen s tem, da se pravilno zvit prekurzor heterolognega proteina po ekspresiji nahaja v inkluzijskih telesih.
2. Postopek pridobivanja proteinov po zahtevku 1, označen s tem, da so heterologni proteini izbrani iz skupine, ki obsega: G-CSF, GM-CSF, M-CSF, EGF, HSA, DNAzo, FGF, TNF-alfa, TNF-beta, interferone in interleukine.
3. Postopek pridobivanja proteinov po zahtevku 1, označen s tem, da je izbrani heterologni protein G-CSF.
4. Postopek pridobivanja proteinov po zahtevku 1, označen s tem, da ekspresija poteka v organizmih, ki so izbrani iz skupine, ki sestoji iz bakterij in kvasovk.
5. Postopek pridobivanja proteinov po zahtevku 4, označen s tem, da ekspresija poteka v bakteriji *E. coli*.
6. Postopek pridobivanja proteinov po zahtevku 1, označen s tem, da vključuje način vodenja biosinteze, ki vključuje enega ali več parametrov, ki so izbrani iz skupine: temperature gojenja, sestave gojišča, načina indukcije, principa vodenja fermentacije in ko-ekspresije pomožnih proteinov.
7. Način vodenja biosinteze po zahtevku 6, označen s tem, da je temperatura gojenja med 20°C in 30°C.
8. Način vodenja biosinteze po zahtevku 7, označen s tem, da je temperatura gojenja okoli 25°C.
9. Način vodenja biosinteze po zahtevku 6, označen s tem, da je induktor izbran iz skupine, ki obsega IPTG, laktozo in NaCl.
10. Način vodenja biosinteze po zahtevku 9, označen s tem, da je izbrani induktor IPTG.
11. Način vodenja biosinteze po zahtevku 10, označen s tem, da je koncentracija IPTG v območju med 0.1 mM in 1 mM.
12. Način vodenja biosinteze po zahtevku 11, označen s tem, da je koncentracija IPTG okoli 0.4 mM.
13. Način vodenja biosinteze po zahtevku 9, označen s tem, da se induktor doda na začetku fermentacije.
14. Način vodenja biosinteze po zahtevku 6, označen s tem, da je princip vodenja biosinteze izbran iz skupine, ki obsega vodenje fermentacije po principu šaržnih

fermentacij, vodenje fermentacije po principu šaržnih fermentacij z dohranjevanjem in vodenje fermentacije v stresani kulturi.

15. Način vodenja biosinteze po zahtevku 14, označen s tem, da je izbrani princip vodenja fermentacije vodenje fermentacije po principu šaržnih fermentacij.
16. Način vodenja biosinteze po zahtevku 6, označen s tem, da je gojišče celic izbrano iz skupine, ki obsega GYST/amp100, GYSP/amp100, LYSP/amp100, LYST/amp100, LBON/amp100 in GYSPON/amp100.
17. Način vodenja biosinteze po zahtevku 16, označen s tem, da je izbrano gojišče GYST/amp100.
18. Način vodenja biosinteze po zahtevku 6, označen s tem, da so dodatki, ki delujejo stresno, izbrani iz skupine, ki obsega etanol in propanol.
19. Postopek pridobivanja proteinov po zahtevku 6, označen s tem, da dodatno vključuje spiranje inkluzijskih teles.
20. Spiranje inkluzijskih teles po zahtevku 19, označeno s tem, da se spiranje izvede s pripravki, ki so izbrani iz skupine, ki obsega Tris/HCl pufer, fosfatni pufer, acetatni pufer, citratni pufer in vodo.
21. Spiranje inkluzijskih teles po zahtevku 20, označeno s tem, da je koncentracija pufrov izbrana v območju med 1 mM in 10 mM.
22. Spiranje inkluzijskih teles po zahtevku 19, označeno s tem, da je izbrani pripravek voda.
23. Postopek pridobivanja proteinov po zahtevku 19, označen s tem, da dodatno vključuje raztapljanje inkluzijskih teles.
24. Raztapljanje inkluzijskih teles po zahtevku 23, označeno s tem, da se za raztapljanje inkluzijskih teles uporabljajo topila, ki so izbrana iz skupine, ki sestoji iz uree v denaturirajočih koncentracijah (1-2 M), N-lauroil sarkozina v nedenaturirajočih koncentracijah (0.05 - 0.2% (m/v)), nizkih koncentracij Zwittergentov, non-detergent sulfobetainov, betaina, sarkozina, karbamoil sarkozina, taurina, DMSO in višjih koncentracij nekaterih pufrov, izbranih iz skupine, ki sestoji iz HEPES, HEPPS, MES, ACES, MES. N-lauroil sarkozina, non-detergent sulfobetainov in DMSO.
25. Raztapljanje inkluzijskih teles po zahtevku 24, označeno s tem, da je izbrano topilo za raztapljanje N-lauroil sarkozin.

26. Raztapljanje inkluzijskih teles po zahtevku 25, označeno s tem, da je koncentracija N-lauroil sarkozina v območju med 0.1% - 0.25%.
27. Raztapljanje inkluzijskih teles po zahtevku 26, označeno s tem, da je koncentracija N-lauroil sarkozina okoli 0.2%.
28. Postopek pridobivanja biološko aktivnega G-CSF, označen s tem, da so izbrani parametri za način vodenja biosinteze:

- temperatura gojenja: okoli 25°C
- sestava gojišča: GYST/amp100
- vodenje fermentacije: šaržni princip fermentacije
- indukcija: IPTG s koncentracijo okoli 0.4 mM
- način indukcije: začetna indukcija

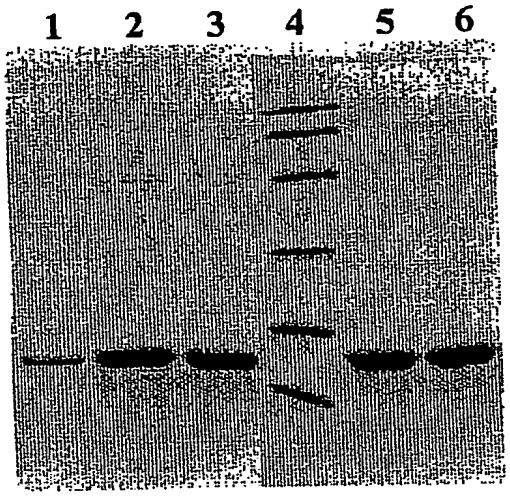
29. Postopek pridobivanja biološko aktivnega G-CSF po zahtevku 28, označen s tem, da nadalje vključuje spiranje inkluzijskih teles z vodo.
30. Postopek pridobivanja biološko aktivnega G-CSF po zahtevku 29, označen s tem, da nadalje vključuje raztapljanje inkluzijskih teles z N-lauroil sarkozinom s koncentracijo okoli 0.2%.

Lek farmacevtska družba d. d.

Izvleček

Izum se nanaša na nov postopek pridobivanja biološko aktivnega heterolognega proteina, ki vključuje vodenje biosinteze heterolognega proteina na tak način, da se izloča pravilno zvit prekurzor heterolognega proteina že v inkluzijksih telesih. Izum dodatno vključuje spiranje in raztapljanje inkluzijskih teles.

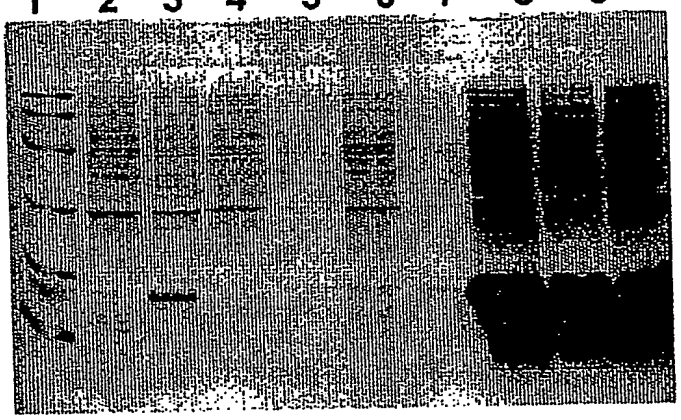
Slika 1



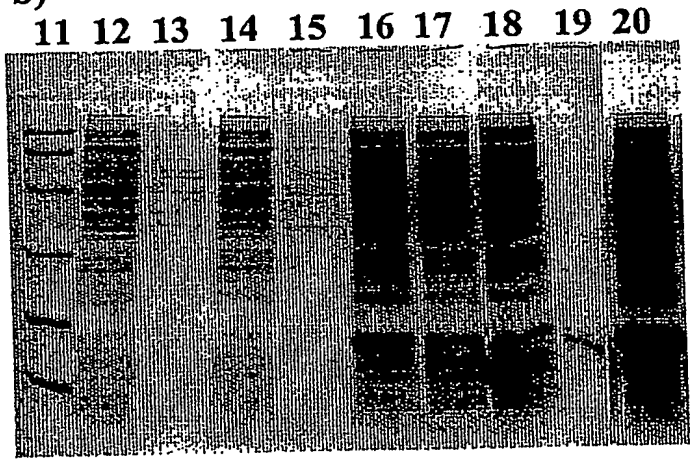
Lek farmacevtska družba d. d.

Slika 2

a)

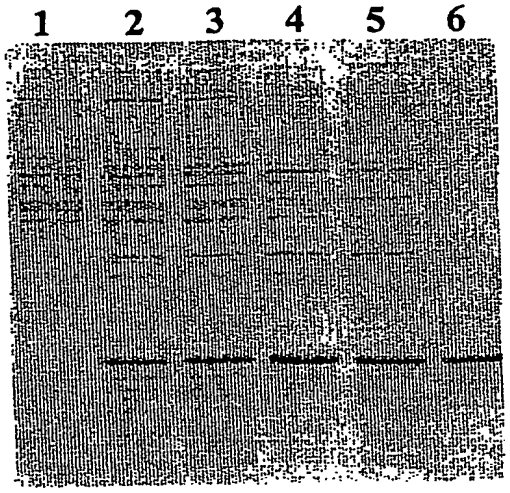


b)



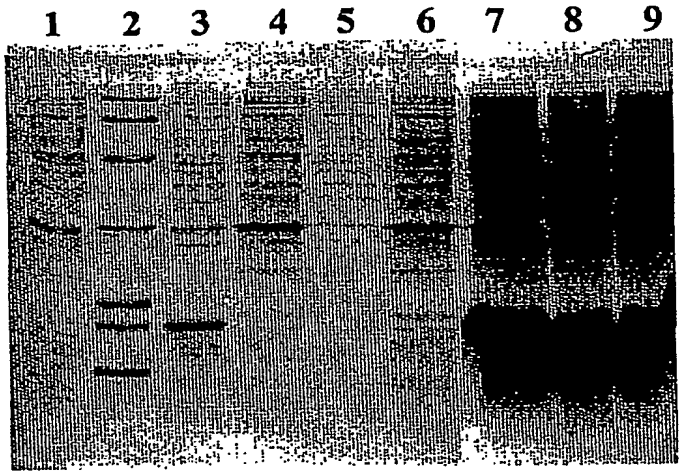
Lek farmacevtska družba d. d.

Slika 3



Lek farmacevtska družba d. d.

Slika 4



Lek farmacevtska družba d. d.

REPUBLIC OF SLOVENIA
Ministry of Economic Affairs

SLOVENIAN INTELLECTUAL PROPERTY OFFICE

Certificate

Slovenian Intellectual Property Office hereby certifies that the document annexed hereto is a true copy of the patent application, as follows:

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Process for the preparation of inclusion bodies with high proportion of correctly folded precursor of a heterologous protein

Ljubljana, 13 September 2002

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REPUBLIC OF SLOVENIA
MINISTRY OF SCIENCE AND TECHNOLOGY

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1. Address for correspondence: Lek Pharmaceuticals d.d. Industrial Property Verovškova 57 1526 Ljubljana Slovenia Telephone: 01 580 23 70 Fax: 01 580 21 23 Code: bk/911	Request for grant of a patent *(for official use only)* Date of application receipt: 31 July 2002 Application number: P - 200200187
2. Applicant (Family name followed by given name and address; for a legal entity, full official designation Lek Pharmaceuticals d.d. Industrial Property Verovškova 57 1526 Ljubljana Slovenia	
3. Representative:	Registration No.:
4. Inventor (Family name followed by given name and address): Lenart Viktor Logatec, Dol 3 1370 Logatec	
5. Title of invention: Process for the preparation of inclusion bodies with high proportion of correctly folded precursor of a heterologious protein	
6. Claimed priority right:	
7. Additional requests: ☐ application for a shortened duration patent ☐ preliminary publication after the expiry of ___months ☐ application is divided form the application no.:___	
8. Statements: ☐ statement of common representative	

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9. Enclosures:

- x Description of the invention, having 27 pages
- x Patent claim (claims), having 3 pages; number of claims: 30
- x Schemes (if required for patent description); number of sheets: 4
- x Abstract
- ☐ Voucher for the settlement of fees
- ☐ Declaration of depositing the biological material if it is about an which cannot be described
- ☐ Authorisation to the representative
- ☐ General authorisation to the representative is deposited in the office under number: .....
- ☐ Declaration of priority right
- ☐ Information of additional applicants
- x Information of additional inventors
- ☐ Presentation of nucleotide or amino acid sequence in the description
- x Application was previously faxed or mailed in electronic form
- ☐ _____

Alenka Košak

_____

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Title of the invention

Process for the preparation of inclusion bodies with high proportion of correctly folded precursor of a heterologous protein

Field of the invention

The present invention relates to a new process for the production of biologically active granulocyte-colony stimulating factor (G-CSF) characterised by the correctly folded precursor of G-CSF found already in the inclusion bodies.

G-CSF belongs to the group of colony stimulating factors which regulate the differentiation and proliferation of hematopoietic precursor cells and have a key role in activation of mature neutrophils. G-CSF is used in medicine in the field of hematology and oncology.

Two types of G-CSF are available on the market: a glycosylated form (lenograstim) which is produced by using the expression in mammalian cells and a nonglycosylated form (filgrastim) which is produced by using the expression in a bacterium *Escherichia coli* (*E. coli*).

Summary of the invention

The essential feature of the present invention is the process for production of biologically active G-CSF. The process comprises the way of performing the biosynthesis in such a way that it enables the secretion of the correctly folded precursor of G-CSF already in the inclusion bodies.

Such way of performing the biosynthesis involves one or more parameters selected from the group comprising: cultivation temperature, induction mode,

principle of performing the fermentation, composition of cultivation medium and co-expression of auxiliary proteins.

The process for production of biologically active G-CSF of the present invention further comprises washing and solubilisation of inclusion bodies and enables the direct isolation of biologically active proteins without use of denaturants or the renaturation process.

Background of the invention

Human granulocyte colony-stimulating factor (hG-CSF) belongs to hematopoetic growth factors which has a decisive role in the formation of neutrophils. Today, two forms for clinical use are on the market: lenograstim which is glycosylated and is produced in a mammalian CHO cell line (Holloway CJ (1994) *Eur J Cancer* 30A Suppl 3:S2-S6., EP 169566), and filgrastim which is nonglycosylated and is produced following its expression in a bacterium *E. coli* (EP 237545).

For intracellular production of heterologous G-CSF in a bacterium *E. coli*, the protein is always secreted in the form of insoluble inclusion bodies. In experiments of secretion into *E. coli* periplasm, G-CSF is secreted either in the form of inclusion bodies (Lei S.P et al (1987) *J Bacteriol* 169:4379-4383; Chung BH et al (1998) *J Ferment Bioeng* 85:443-446) or there has been no report on biological activity of G-CSF produced in this way (Jeong KJ & Lee SY (2001) *Protein Expression and Purification* 23: 311-318).

From the aforementioned, it is clear that in almost all described experiments of isolation of G-CSF from a bacterium *E. coli* in prior art G-CSF is found in inclusion bodies.

Several examples of inclusion bodies produced in yeast have been described, however, there has been no report on G-CSF.

For production of correctly folded biologically active proteins from insoluble inclusion bodies, inclusion bodies should be isolated from the cells, washed and solubilised, and then *in vitro* renaturation should be performed. *In vitro* renaturation is an additional step in the process of large-scale isolation of proteins.

Processes for the production of recombinant proteins from inclusion bodies comprise lysis and disruption of the cells followed by centrifuging. The pellet comprising a large proportion of inclusion bodies is usually washed with detergents. For isolation of G-CSF, washing of inclusion bodies with 1% deoxycholate (Zsebo KM et al (1986) *Immunobiology* 172:175-184. EP237545; US5849883) or with a solution of 1.0 M NaCl with added 0.1% Tween 80 (Gelunaite L et al (2000) *J Chromatogr A* 904:131-143) has been reported to be convenient.

A further step in obtaining recombinant proteins is solubilisation of inclusion bodies requiring generally the use of rather strong denaturants. For production of G-CSF the use of 6M GdHCl (Wingfield P et al (1988) *Biochem J* 256:213-218; Zink T et al (1992) *FEBS Lett* 314:435-439.), 7 M urea at pH 7.2 (Kuga T et al (1989) *Biochem Biophys Res Commun* 159:103-111) or 8 M urea pH 8.0 (Yamasaki M et al (1998) *Biosci Biotechnol Biochem* 62:1528-1534), 20 mM Tris/HCl and addition of EDTA and DTT has been described. Kang S-H and co-workers succeeded to solubilise inclusion bodies in 2 M urea in strongly alkaline pH about 12 (Kang SH et al (1995) *Biotechnol Lett* 17:687-692). A similar process is solubilisation in 10 mM HEPES buffer with a short-term increase of pH to 11.5 (Gelunaite L et al (2000) *J Chromatogr A* 904:131-143). Strong denaturants in denaturating concentrations are equally effective for solubilisation, for example, 1% SDS with added 5% ethanol in 50 mM Tris/HCl pH 8.5 (Souza LM et al (1986) *Science* 232:61-65). 2% sarcosyl is often used (Lu HS et al (1992) *J Biol Chem* 267:8770-8777; Lu HS et al (1993) *Protein Expr Purif* 4:465-472; Young DC et al (1997) *Protein Science* 6: 1228-1236; EP237545; US5849883) or 1% sarcosyl alone (Zsebo KM et al (1986) *Immunobiology* 172:175-184). In most cases, solubilisation of inclusion bodies requires the use of strong detergents which are toxic and non-nature-friendly and are serious environmental pollutants. Their use is also uneconomical, because safe removal after the end of the process is an additional cost and is time-consuming.

Although some examples of the separation of protein under denaturating conditions in the first chromatographic step, for example on C4 RP HPLC (Souza LM et al 1986) *Science* 232:61-65; Zsebo KM et al (1986) *Immunobiology* 172:175-184; EP237545) or by using gel filtration (Wingfield P et al (1988) *Biochem J* 256:213-218;

US4999291) are described, in the majority of examples chromatographic separation runs after preliminary *in vitro* renaturation.

Solubilisation of inclusion bodies in strong denaturants is followed by *in vitro* renaturation of proteins. *In vitro* renaturation of G-CSF is achieved by dialysis against the buffer with a smaller concentration of a denaturant (Wingfield P et al (1988) *Biochem J* 256:213-218), by dialysis against the buffer without a denaturant (Kuga T et al (1989) *Biochem Biophys Res Commun* 159:103-111), in the buffer with added 0.8 M arginine (Zink T et al (1992) *FEBS Lett* 314:435-439) or by gel filtration (US4999291; D.C. Young et al (1997) *Protein Science* 6: 1228-1236). After solubilisation in the denaturing concentrations of sarcosyl, oxidative folding is achieved with the addition of $CuSO_4$ in low concentrations during long-term stirring at room temperature, followed by removal of a detergent by using a strong base ion exchanger (US5849883). In the cases the solubilisation runs under extremely alkaline conditions, protein is spontaneously renaturated by restoring a lower pH (Kang SH et al (1995) *Biotechnol Lett* 17:687-692). All described methods for *in vitro* renaturation are complex and time-consuming. Large-scale dilution requires the large capacity apparatus and consequently the higher initial investment (larger space, larger apparatus ....), and for the production higher operating costs (power, water, buffers ...).

Improved *in vitro* renaturation is achieved in fusion protein wherein and oligopeptide hydrophilic tag and restriction site for IgA protease are added to the N-terminus (EP0500108).

In the literature an increase in proportion of soluble protein in the cytoplasm has been reported (Weickert MJ et al (1997), Appl and Environmental Microbiology, 63:4313-4320; Bhandari P & Gowrishankar J (1997) J. Bacteriol, 179: 4403-4406; Zhang Y et al (1998) Protein Expr Purif, 12: 159-165; Thomas JG and Baneyx F (1996) J of Biological Chemistry, 271:11141-11147; Kapust RB and Waugh DS (1999) Protein Science, 8: 1668-1674; Davis GD et al (1999) Biotech and Bioeng 65: 382-388), but there has been no report on the secretion of G-SCF in the cytoplasm instead of its secretion in inclusion bodies.

To our knowledge, there has so far been no reports in the patent and scientific literature on increase in proportion of the correctly folded heterologous protein already in the inclusion bodies.

In the case of G-CSF, *in vitro* renaturation is reported as the first condition and thus the only possible way for the production of biologically active protein (Rudolph R (1996) in *Protein Engineering: Principles and Practice* (Cleland JL and Craik CS eds) pp 283-298, Wiley-Liss, Inc., New York).

Description of drawings

Figure 1: Protein composition in the inclusion bodies

Method: SDS-PAGE (4% stacking, 15% separating gel, stained with Coomassie brilliant blue).

The conditions for the preparation of inclusion bodies: cultivation temperature 25°C, performing of fermentation in a batch mode, induction with 0.4 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at $OD_{600nm} \approx 6.0$ or at the beginning, washing with cold water or cold 10 mM TrisHCl/pH=8.0 buffer, solubilisation in 0.2 % sarcosyl.

Lane 1: hG-CSF standard 0.6 μg

Lane 2: induction at $OD_{600nm} \approx 6.0$, inclusion bodies washed with water

Lane 3: induction at $OD_{600nm} \approx 6.0$, inclusion bodies washed with 10 mM TrisHCl/pH=8.0 buffer.

Lane 4: molecular weight standards (LMW - BioRad)

Lane 5: induction at the beginning, inclusion bodies washed with water

Lane 6: induction at the beginning, inclusion bodies washed with 10 mM TrisHCl/pH=8.0 buffer.

Figure 2: Comparison of the solubilisation of the inclusion bodies regarding the cultivation temperature (25°C or 37°C).

Method: SDS-PAGE (4% stacking, 15% separating gel, stained with Coomassie brilliant blue).

The conditions for the preparation of inclusion bodies: cultivation temperature: 25°C (lanes 4-10) and 37°C (lanes 12-18, 20), performing of fermentation in a batch mode, induction with 0.4 mM IPTG at the beginning, washing with cold water or cold 10 mM TrisHCl/pH=8.0 buffer, 1% Na-deoxycholate, 1% Triton X-100, 2 M urea or 8 M urea.

Lane 1: molecular weight standards (LMW - BioRad)

Lane 2: the soluble fraction of proteins (homogenate supernatant)

Lane 3: total proteins

Lane 4: solubilised proteins after the first washing of the inclusion bodies with water

Lane 5: solubilised proteins after the second washing of the inclusion bodies with water.

Lane 6: solubilised proteins after the first washing of the inclusion bodies with 10 mM TrisHCl/pH=8.0

Lane 7: solubilised proteins after the second washing of the inclusion bodies with 10 mM TrisHCl/pH=8.0

Lane 8: solubilised proteins after washing of the inclusion bodies with 1% Na-deoxycholate

Lane 9: solubilised proteins after washing of inclusion bodies with 2 M urea

Lane 10: solubilised proteins after washing of inclusion bodies with 1% Triton-X100

Lane 11: molecular weight standards (LMW - BioRad)

Lane12: solubilised proteins after the first washing of the inclusion bodies with water

Lane 13: solubilised proteins after the second washing of inclusion bodies with water

Lane 14: solubilised proteins after the first washing of inclusion bodies with 10 mM TrisHCl/pH=8.0

Lane 15: solubilised proteins after the second washing of inclusion bodies with 10 mM TrisHCl/pH=8.0

Lane 16: solubilised proteins after washing of inclusion bodies with 1% Na-deoxycholate

Lane 17: solubilised proteins after washing of inclusion bodies with 2 M urea

Lane 18: solubilised proteins after washing of inclusion bodies with 1% Triton-X100
Lane 19: standard hG-CSF, 0.6 µg
Lane 20: solubilised proteins after washing of inclusion bodies with 8 M urea

Figure 3: Composition of proteins in the inclusion bodies

Method: SDS-PAGE (4% stacking, 15% separating gel, stained with Coomassie brilliant blue).

The conditions for the preparation of inclusion bodies: production strain BL21-SI pET3a/P-Fopt5, cultivation temperature 25°C, fermentation in shake flasks, non-induced culture or induction with 1.2 M NaCl at $OD_{600nm} \approx 0.5$ or with the induction at the beginning.

Lane 1: BL21 (SI) pET3a/P-Fopt5, non-induced culture
Lane 2: BL21 (SI) pET3a/P-Fopt5, induction with 1.2 M NaCl at $OD_{600nm} \approx 0.5$
Lane 3: BL21 (SI) pET3a/P-Fopt5, induction with 1.2 M NaCl at $OD_{600nm} \approx 0.5$
Lane 4: BL21 (SI) pET3a/P-Fopt5, induction with 1.2 M NaCl at the beginning
Lane 5: BL21 (SI) pET3a/P-Fopt5, induction with 1.2 M NaCl at the beginning
Lane 6: rhG-CSF 0.6 µg

Figure 4: Comparison of the solubility of the inclusion bodies with regard to washing with different solvents

Method: SDS-PAGE (4% stacking, 15% separating gel, stained with Coomassie brilliant blue).

The conditions for the preparation of inclusion bodies: strain BL21(SI) pET3a/P-Fopt5, cultivation temperature 25°C, fermentation in shake flasks, induction with 1.2 M NaCl, washing with water, 10 mM TrisHCl/pH=8.0, 1 % Na-deoxycholate, 1 % Triton X-100 or 2 M urea.

Inclusion bodies were prepared in shake flasks at 25°C, induced with 1.2 mM NaCl added in the medium at the beginning.

Lane 1: soluble fraction of the proteins (homogenate supernatant)
Lane 2: molecular weight standards (LMW - BioRad)
Lane 3: total proteins
Lane 4: solubilised proteins after the first washing of inclusion bodies with water

Lane 5: solubilised proteins after the second washing of inclusion bodies with water
Lane 6: solubilised proteins after the first washing of the inclusion bodies with 10 mM TrisHCl/pH=8.0
Lane 7: solubilised proteins after washing of inclusion bodies with 1% Na-dexycholate
Lane 8: solubilised proteins after washing of inclusion bodies with 2 M urea
Lane 9: solubilised proteins after washing of inclusion bodies with 1% Triton-X100

Description of the invention

It has been surprisingly found that by the process for the production of biologically active G-CSF of the present invention the biosynthesis can be performed in such a way that a correctly folded precursor of G-CSF is accumulated already in the inclusion bodies.

Although the inclusion bodies described in the patent and scientific literature present insoluble compact aggregates of incorrectly folded or partially correctly folded proteins, in the present invention it has been achieved that the correctly folded precursor of G-CSF is accumulated in inclusion bodies. The inclusion bodies which comprise the correctly folded precursor of G-CSF are more soluble than the slightly soluble inclusion bodies found in the art. Higher solubility enables easier and less time- consuming production of G-CSF from inclusion bodies. Therefore, the yields are high, being useful, practical and economical in the large-scale production.

The term 'correctly folded precursor' of the protein (G-CSF), as used herein, refers to a protein (G-CSF) having the correct conformation (three-dimensional structure), but the disulphide bridges are not formed yet.

We suppose that the correctly folded precursor of G-CSF is somehow trapped within other insoluble aggregates which form inclusion bodies, therefore, only elimination from these aggregates is required. The elimination is performed by washing and solubilisation of inclusion bodies. For such elimination only weak solvents can be used, and not strong denaturating agents conventionally used for solubilisation of inclusion bodies. The process of elimination of G-CSF precursor

triggers the spontaneous oxidation (due to presence of air oxygen...) and causes the spontaneous formation of the intramolecular disulphide bridges. The biologically active G-CSF is therefore obtained.

The term 'biologically active G-CSF', as used herein, refers to G-CSF with the biological activity which is the same or higher than $1x10^8$ IU/mg, and is determined by the cell proliferation method as described in Example 12.

In comparison with other known procedures of production of heterologous proteins which, after expression, are accumulated in inclusion bodies, the procedure of the present invention is better from the economical viewpoint. Instead of washing with strong detergents only washing with water or buffer is needed and the solubilisation runs under weak conditions and not in the presence of strong denaturants or detergents in high concentrations. Further there is no need for *in vitro* renaturation procedures normally required for refolding.

The term 'heterologous protein', as used herein, refers to the protein which is foreign to the organism in which expression is performed.

The term 'renaturation', as used herein, refers to conversion of denaturated proteins to conformation the proteins had before denaturation.

The term '*in vitro* renaturation', as used herein, refers to the renaturation which is performed outside the organism.

The term 'denaturation', as used herein, refers to a process in which the conformation of the protein (three-dimensional structure) is changed but the primary structure (amino acid chain, peptide links) of the protein remains unchanged.

The term 'denaturant', as used herein, refers to the solution in which the conformation of proteins is not preserved. Biological activity of the proteins in the presence of denaturants is changed and is not preserved.

The term 'aggregate', as used herein, refers to the cluster of the molecules which are mutually linked predominantly with hydrophobic as well as with other bonds (as for example disulphide). These molecules are not biologically active.

The process for production of biologically active G-CSF of the present invention which enables formation of the correctly folded precursor of G-CSF already in the in inclusion bodies and obtaining biologically active G-CSF directly from inclusion bodies, comprises the principle of performing the biosynthesis which

comprises one or a combination of several parameters which are selected from the group comprising:

- cultivation temperature,
- induction mode,
- performing of fermentation,
- cultivation medium composition and
- co-expression of auxiliary proteins.

The term 'biosynthesis', as used herein, refers to production of a heterologous protein by using microorganisms.

The term 'cultivation', as used herein, refers to the growth of microorganisms under controlled conditions submersed or on solid supports with the provided source of substrates needed for growth of microorganisms.

The term 'fermentation', as used herein, refers to cultivation of microorganisms under submersed conditions in a bioreactor (fermentor) or shake flasks.

The process for production of biologically active G-CSF of the present invention further comprises washing and solubilisation of inclusion bodies and enables the isolation of biologically active G-CSF without use of denaturants or *in vitro* renaturation process.

The essential feature of the process for production of biologically active G-CSF of the present invention is that the way of performing the biosynthesis comprises the parameters which enable the regulation of the composition of inclusion bodies in such a way that the proportion of the correctly folded protein molecules of G-CSF (precursor of G-CSF) is increased. High accumulation of G-CSF does not simultaneously mean a high proportion of the correctly folded precursor of G-CSF. The process for production of biologically active G-CSF of the present invention also enables to find an optimal ratio between accumulation of G-CSF and a proportion of correctly folded precursor of G-CSF.

This enables a high yield large-scale production of biologically active G-CSF.

The term 'accumulation of G-CSF', as used herein, refers to a proportion of G-CSF obtained by using the heterologous expression of the gene for G-CSF with regard to total proteins which are present after expression.

The term 'heterologous expression', as used herein, refers to the expression of those genes which are foreign to the organism in which the expression is performed.

The term 'proportion of correctly folded precursor of G-CSF', as used herein, refers to proportion of the correctly folded precursor of G-CSF with respect to total G-CSF proteins (correctly, partly correctly, incorrectly folded).

The term 'proportion of biologically active G-CSF', as used herein, refers to proportion of the correctly folded precursor of G-CSF after washing and solubilisation of inclusion bodies. After solubilisation the correctly folded precursor of G-CSF is rendered biologically active due to spontaneous oxidation and, hence, spontaneous formation of the disulphide bonds.

The process for production of biologically active G-CSF of the present invention enables the preparation of G-CSF which is suitable for clinical use in human and veterinary medicine.

It can be used for the preparation of human G-CSF and other mammalian G-CSF, as well as for the preparation of G-CSF derivatives, such as methionyl G-CSF (Met-G-CSF), enzymatically and chemically modified (such as, for example pegylated) G-CSF, G-CSF analogs and fusion proteins which comprise G-CSF.

The process for production of biologically active G-CSF of the present invention can be also used for production of other biologically active heterologous proteins which are accumulated in the form of inclusion bodies after expression. These proteins are selected from the group comprising: interferons (IFN), such as INF-beta 1b, IFN-beta 2b, IFN-gamma 1b, interleukins (IL), such as IL-2 and IL-4, granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor, (M-CSF), epidermal growth factor (EGF), human serum albumin (HSA), deoxyribonuclease (DNAse), fibroblast growth factor (FGF), tumour necrosis factor alpha (TNF alpha) and tumour necrosis factor beta (TNF-beta) and further comprises all other partially hydrophobic proteins with not too many disulphide bonds which (in great proportion) can be spontaneously folded in the correct 3D structure.

The process for production of biologically active G-CSF of the present invention can be used in the case of formation of the inclusion bodies after expression irrespective of the organism used as the host for expression. As host

organisms can be used those selected from the group comprising primarily bacteria and yeast. Of bacterial systems *E. coli* and *Streptomyces* spp., and of yeast systems conventional yeast strains such as *Saccharomyces cerevisiae*, and unconventional yeast strains such as *Pichia pastoris, Hansenula polymorpha, Candida utilis* and others are most frequently employed.

The way of performing the biosynthesis of G-CSF of the present invention comprises the parameters which are selected from the group: cultivation temperature, cultivation medium composition, induction mode, principles of performing the fermentation and co-expression of auxiliary proteins. By optimising the individual parameter of the aforementioned parameters, it possible to increase substantially the proportion of the correctly folded precursor of G-CSF, and the combination of the parameters enables additionally higher proportion.

Cultivation temperature

It has been surprisingly found that the decreasing of cultivation temperature enables the secretion of a correctly folded precursor of G-CSF already in the inclusion bodies. Normal optimal cultivation temperature of bacterial cells is 37°C. In the present invention, it has been found that preferable temperature for the secretion of the correctly folded precursor of G-CSF already in inclusion bodies is significantly lower than 37°C, namely, between 20°C and 30°C. The most preferable temperature is about 25°C.

Induction mode

It has been surprisingly found that the proportion of the correctly folded precursor of G-CSF in inclusion bodies also depends on the induction mode. Induction is possible with the addition of an inductor which is selected from the group: IPTG, lactose and NaCl. Preferred induction is with IPTG. Proportion of the correctly folded precursor of G-CSF in inclusion bodies also depends on the concentration of the inductor. When IPTG is added the chosen concentration is in the range from 0.1 mM to 1 mM. Preferred concentration is about 0.4 mM. When NaCl is used the chosen concentration is in the range from 0.3 M to 1.3 M. Preferred concentration is about 1.2 M. When lactose is used the chosen concentration is in the range between 1 and 10 g/l, the most preferred concentration is between about 2 and about 4 g/l. Proportion of the correctly folded precursor of G-CSF in inclusion bodies also

depends on the induction mode (the time of cultivation). The inductor can be added at the beginning of fermentation (instantaneously), which is preferable in case of IPTG, NaCl or lactose. IPTG and NaCl can be also added in later steps of the fermentation at OD_{600nm} about 6.0 (OD is the measure for the bacterial count).

Principle of performing the fermentation

It has been surprisingly found that proportion of the correctly folded precursor of G-CSF in inclusion bodies also depends on the performing the fermentation. It is selected from the group comprising: fermentation in a bioreactor and performing the fermention in a shake flasks. Performing the fermentation in a bioreactor is preferable which comprises performing the fermentation in a batch mode and performing the fermentation in a fed-batch mode. Preferred principle of performing the fermentation is a batch mode where the high productivity of biomass with highly soluble inclusion bodies is obtained.

Co-expression of auxiliary proteins

Further it has been found that proportion of the correctly folded precursor of G-CSF in inclusion bodies also depends of the additions capable of causing stress. These additives trigger co-expression of stress proteins and are selected from the group comprising various concentrations of ethanol and propanol in the range from 1% to 5% (v/v). Use of ethanol and propranol at a concentration of 3% (v/v) is most preferable.

Cultivation medium composition

Further it has been surprisingly found that proportion of the correctly folded precursor of G-CSF in inclusion bodies also depends on the cultivation medium composition. Media are selected from the group comprising: GYST/amp100 (20 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 10 g/l glucose, metals in traces, 100 mg/l ampicillin), GYSP/amp100 (20 g/l phytone, 5 g/l yeast extract, 10 g/l NaCl, 10 g/l glucose, metals in traces, 100 mg/l ampicillin) and LYSP/amp100 (20 g/l phytone, 5 g/l yeast extract, 10 g/l NaCl, 6 g/l glycerol, 2 g/l or 4 g/l lactose, metals in traces, 100 mg/l ampicillin), LYST/amp100 (20 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, 6 g/l glycerol, 2 g/l or 4 g/l lactose, metals in traces, 100 mg/l ampicillin), LBON/amp100 (10 g/l phytone, 5 g/l yeast extract, 100 mg/ml ampicillin), GYSPON/amp100 (20 g/l phytone, 5 g/l yeast extract, 10 g/l glucose, metals in traces, 100 mg/l ampicillin)

(Metals in traces: ($FeSO_4x7H_2O$: 40 mg/l, $CaCl_2x2H_2O$: 40 mg/l, $MnSO_4xnH_2O$: 10 mg/l, $AlCl_3x6H_2O$: 10 mg/l, $CoCl_2x6H_2O$: 4 mg/l, $ZnSO_4x7H_2O$: 2 mg/l, $NaMoO_4x2H_2O$: 2 mg/l, $CuSO_4x5H_2O$: 1 mg/l, H_3BO_3: 0.5 mg/l)). Preferably GYST/amp 100 medium is used.

Washing of inclusion bodies

A higher proportion of the biologically active protein in inclusion bodies leads to higher solubility of inclusion bodies. If the inclusion bodies are then washed with detergents, high proportion of correctly folded precursor of G-CSF is washed with the washing solution. Therefore, washing can be performed with different solutions which are selected from the group comprising water and different buffers in very low concentrations (1mM to 10mM), for example, buffer Tris/HCl, phosphate buffer, acetate buffer, citrate buffer. Most preferred is washing with water.

Solubilisation of inclusion bodies

Higher solubility of inclusion bodies which occurs due to higher solubility of a correctly folded precursor of heterologous protein in inclusion bodies indicates that the solubilisation is performed under 'smooth' conditions, without the addition of strong denaturants or denaturating concentrations of detergents. For the solubilisation of inclusion bodies, the solvents to be used are selected from the group comprising: urea in non-denaturing concentrations (1–2 M), N-lauroyl sarcosine in non-denaturing concentrations (0.05–0.25% (m/v)), low concentrations of Zwittergents, different non-detergent sulfobetains (NDSB), betain, sarcosine, carbamoil sarcosine, taurine, dimethylsulfoxide (DMSO) and higher concentrations of some buffers, for example: HEPES, HEPPS, MES, ACES, MES. Preferably N-lauroyl sarcosine, NDSB and DMSO are used. Most preferably N-lauroyl sarcosine in the concentration range 0.1% to 0.25% (m/v) is used.

Cultivation temperature about 25°C, performing of fermentation in a batch mode, cultivation medium GYST/amp100, induction mode at the beginning of the fermentation, IPTG induction with the concentration of about 0.4 mM, washing with water and solubilisation of inclusion bodies in N-lauroyl sarcosine at the concentration of about 0.2% are the parameters where the proportion of correctly folded precursor of G-CSF (and consequently the proportion of biologically active G-

CSF) and the accumulation of G-CSF are high. With these parameters, the composition of the resulting proteins enables the G-CSF obtained in this manner to be used directly in further isolation procedures, especially as the loading solution for immobilised metal affinity chromatography - IMAC. This process can be used in the large-scale production of G-CSF with high yields.

Examples

Example 1: Influence of the cultivation temperature on the proportion of the correctly folded precursor of G-CSF in inclusion bodies and on the concentration of total proteins after solubilisation of the inclusion bodies in 0.2% sarcosyl

Human gene for G-CSF was modified for high expression in a bacterium *E. coli* (Fopt5). In the expression system *E. coli* BL21 (DE3) with pasmid pET3a, the level of accumulation of G-CSF is achieved accounting for over 50% of all cell proteins. *E. coli* culture BL21(DE3) pET3a-Fopt5 from the strain bank at -70^0C was inoculated at in a ratio 1 to100 in LBG/amp medium (10 g/l tryptone, 5g/l yeast extract, 10 g/l NaCl, 2.5 g/l glucose, 100 mg/l ampicillin) and the culture was cultivated for 10 hours on a shaker at 25°C, 160 rpm. Two to 4 ml of this culture was inoculated in 40 ml GYST/amp100 medium to which the IPTG inductor to the final concentration of 0.4 mM was added at the beginning of the fermentation (initial $OD_{\lambda 600nm}$ of the culture was 0.385). The culture was then cultivated on a shaker (shake flasks) at three different temperatures and at 160 rpm to the exponential phase of growth:

- at 25°C: 22 hours, final $OD_{\lambda 600nm}$ of the culture was 15.2 ± 0.5;
- at 37°C: 15 hours, final $OD_{\lambda 600nm}$ of the culture was 12.2 ± 0.4;
- at 42°C: 15 hours, final $OD_{\lambda 600nm}$ of the culture was 6.75 ± 0.2.

After completed cultivation, the resulting cultures were centrifuged for 10 minutes at 5000 rpm, washed with a 4–5-fold volume of 10 mM TrisHCl/pH=8.0 and the biomass was transferred into the new centrifuge tubes and centrifuged again for 10 minutes at 5000 rpm. The biomass was resuspended in a 5-fold volume of 10 mM TrisHCl/pH=8.0 buffer and sonicated 12 x for 1 minute with a submersed probe (duty cycle: 60%, power: 7, pulses: 2 s^{-1}). After sonification the broken cells were centrifuged again for 30 minutes at 5000 rpm. The pellet with inclusion bodies was solubilised under non-denaturating conditions in a 50-fold volume of 0.2 % N-lauroyl

sarcosine in 40 mM TrisHCl/pH=8.0, solubilisation was left to run overnight 16 to 18 hours at room temperature under shaking at 50 rpm. After solubilisation, the protein concentration was determined according to the Bradford method using pure hMet-G-CSF as a standard. The concentrations were about 1 mg/ml for 42°C and 37°C and between 2 and 3 mg/ml for 25°C. The total protein concentrations after solubilisation of the inclusion bodies in 0.2% N-lauroyl sarcosine and the proportion of correctly folded precursor of G-CSF and biologically active G-CSF, respectively, prepared from the biomass cultivated at different temperatures are shown in Table 1.

The proportion of the correctly folded precursor of G-CSF and biologically active G-CSF, respectively, (after elimination from the inclusion bodies) was determined by measuring the biologically activity of the solubilised inclusion bodies without removal of N-lauroyl sarcosine (at this dilution sarcosyl does not interefere with).

Cultivation temperature	Concentration of total proteins after solubilisation of inclusion bodies in 0.2 % N-lauroyl sarcosine	Proportion of biologically active G-CSF
25°C	2.7 mg/ml	> 30%
37°C	1.1 mg/ml	> 20%
42°C	1.0 mg/ml	< 1%

Table 1: Concentration of total proteins after solubilisation of inclusion bodies in 0.2% N-lauroyl sarcosine and proportion of the correctly folded precursor of G-CSF or biologically active G-CSF prepared from the biomass cultivated at different temperatures

As evident from the data presented in Table 1, the proportion of the correctly folded precursor of G-CSF in the inclusion bodies was markedly increased with cultivation at decreased temperature. Further, up to 2.5-fold higher concentrations of total proteins were obtained after solubilisation of the inclusion bodies in 0.2 % N-lauroyl sarcosine suggesting that the inclusion bodies prepared this way are essentially more soluble.

Example 2: Influence of the induction mode with IPTG on the accumulation of G-CSF

Induction with 0.4 mM IPTG at the beginning of the fermentation

Previous experiments with different concentrations of IPTG (0.05 mM – 0.4 mM) showed that a sufficiently high and almost the same accumulation of G-CSF was achieved in the range 0.1– 0.4 mM for the biomass with the final $OD_{\lambda 600nm}$ = 20–30.

E. coli culture BL21(DE3) pET3a-Fopt5 from the strain bank at –70°C was inoculated in a ratio 1 to 500 in the LBG/amp medium and cultivated for 14–18 hours on a shaker at 25°C, 150 rpm. This culture was used as an inoculation culture to inoculate the fermentor culture in a ratio 1 to 20 in the production medium GYST/amp to which the IPTG inductor to the final concentration of 0.4 mM was added at the beginning of the fermentation. The fermentation was performed as a batch mode for 20–23 hours at 25°C, 500 rpm and air flow 1 vvm (7 L Chemap fermentor). At the end of the process, the $OD_{\lambda 600nm}$ of the culture was approximately 25.

The biomass was centrifuged for 5 minutes at 5000 rpm (Beckman centrifuge) and frozen for further processing. After SDS-PAGE and staining with Coomassie, the proportion of G-CSF in total proteins was determined by using a densitometric analysis. It ranged between 30% and 40%.

Induction with 0.4 mM IPTG at $OD_{600nm} \approx 6.0$

The fermentation was performed in the manner similar to that for the induction with 0.4 mM IPTG at the beginning of the process, except that the IPTG inductor to the final concentration or 0.4 mM was added when the culture reached $OD_{600nm} \approx 6.0$. The fermentation was performed in a batch mode 18–20 hours at 25°C, 500 rpm and air flow 1 vvm (7 L Chemap fermentor). At the end of the process the $OD_{\lambda 600nm}$ of the culture was approximately 30. The biomass was centrifuged for 5 minutes at 5000 rpm (Beckman centrifuge) and frozen for further processing. After SDS-PAGE and staining with Coomassie, the proportion of G-CSF in total proteins was determined by using the profile densitometric analysis, it ranged between 30% and 40%. The inclusion bodies were isolated from the biomass as described in Example 3. The pellet with the inclusion bodies was washed with cold water or cold 10 mM TrisHCl/pH=8 buffer, centrifuged again for 30 minutes at 10000 rpm and solubilised under non-denaturing conditions with a 50-fold volume of 0.2% N-lauroyl sarcosine in

40 mM TrisHCl/pH=8.0. Solubilisation was left to run overnight for 16 to 18 hours at room temperature under shaking at 100–150 rpm. After solubilisation the concentration of proteins was determined by the Bradford method using pure hMet-G-CSF as a standard.

The concentration of total solubulised proteins ranged between 2 and 4 mg/ml after both induction modes.

Comparison of the proportion of hG-CSF in the inclusion bodies when IPTG is added at the beginning of the fermentation (instantaneous induction) or when OD_{600nm} the of the culture is approx. 6.0

When IPTG is added to the medium at the beginning, the inclusion bodies with very high content of G-CSF and insignificant impurities of other *E. coli* proteins are obtained. In the case of induction at $OD_{600nm} \approx 6.0$, the content of G-CSF in the inclusion bodies is still very high but the content of other *E. coli* proteins is higher and may interfere with the subsequent isolation steps (Figure 1).

Example 3: The influence of temperature on the solubility of inclusion bodies

The solubility of inclusion bodies is much increased when the cells are cultivated at 25°C. Washing with detergents would therefore lead to losses of the correctly folded precursor of G-CSF in the washing solution. However, washing is still needed (at least with water) as otherwise some proteins that may disturb the chromatography remain in the solution.

Preparation of biomass: *E. coli* culture BL21(DE3) pET3a/P-Fopt5 from the strain bank at -70^0C was inoculated in a ratio 1 to 500 to LBG/amp medium and the culture was cultivated 14-18 hours on a shaker at 150 rpm at 25^0C. This culture was used as an inoculation culture to inoculate the fermentor culture in a ratio of 1 to 20 in a production medium GYST/amp to which the IPTG inductor to the final concentration of 0.4 mM was added at the beginning of the fermentation. The fermentation was performed as a batch mode at 500 rpm and air flow 1 vvm (7 L Chemap fermentor) at two different temperatures:

- at 25°C 18–25 hours, at the end of the process the $OD_{\lambda 600nm}$ of the culture was approx. 25.

- at 37°C 8–25 hours, at the end of the process the $OD_{\lambda 600nm}$ of the culture was approx. 28.

Isolation of inclusion bodies: After the completed fermentation, the bacterial pellet was separated from the supernatant by centrifugation at +4°C and 5000 rpm. The wet bacterial pellet was resuspended in a 4-fold volume of buffer X (10 mM Tris/HCl, pH=8,0). The homogenisation of the sample was performed by using ultraturax. The cells were then broken by using the homogeniser EmulsiFlex C-5 (AVESTIN) in one passage at the pressure difference of 10000 to 15000 psi (70 – 110 MPa). After the 30-minute centrifugation at 10000 rpm the supernatant comprising the soluble bacterial *E. coli* proteins was thrown away, and the pellet (inclusion bodies) was frozen at –20°C and was used in inclusion bodies washing and solubilisation experiments.

The following experiments of inclusion bodies washing were performed (Figure 2):

A. Washing of inclusion bodies with water

The determined quantity of inclusion bodies was resuspended in a 10-fold volume of cold water (+4°C), centrifuged for 10 minutes at 10000 rpm at +4°C and the process was repeated with the same volume of cold water. In the supernatants after both washings the amount of proteins was determined according to the Bradford method (using bovine serum albumin (BSA) as a standard). The protein composition was analysed by SDS-PAGE.

Water quality: The water was prepared by using Milli-Q RG (Millipore) apparatus.

B. Washing of inclusion bodies with buffer Y (10mM Tris/HCl, pH 8.0)

The determined quantity of inclusion bodies was resuspended in a 10-fold volume of cold buffer Y (10 mM Tris/HCl, pH 8.0) (+4°C), centrifuged for 10 minutes at 10000 rpm at +4°C and the process was repeated with the same volume of cold water. In the supernatants after both washings the amount of proteins was determined according to the Bradford method (using BSA as a standard). The protein composition was analysed by SDS-PAGE.

C. Washing of inclusion bodies with 1% Na-deoxycholate

The determined quantity of inclusion bodies was resuspended in a 10-fold volume of cold (+4°C) buffer W (50 mM Tris/HCl, pH 9.0 with added 1% Na deoxychyolate, 5 mM dithiothreitol (DTT) and 5 mM EDTA, left for 30 minutes on

ice and centrifuged for 10 minutes at 10000 rpm at +4°C. In the supernatants the amount of proteins was determined according to the Bradford method (using BSA as a standard). The protein composition was analysed by SDS-PAGE.

D. Washing of inclusion bodies with 1 %Triton X-100

The determined quantity of inclusion bodies was resuspended in a 10-fold volume of cold (+4°C) buffer X (10 mM Tris/HCl, pH=8.0) with added 1% Triton X-100, left for 30 minutes on ice and centrifuged for 10 minutes at 10000 rpm at +4°C. In the supernatants the amount of proteins was determined according to the Bradford method (using BSA as a standard). The protein composition was analysed by SDS-PAGE.

E. Washing of inclusion bodies with 2 M urea

The determined quantity of inclusion bodies was resuspended in a 10-fold volume of cold (+4°C) buffer X (10 mM Tris/HCl, pH=8.0) with added 2 M urea, left for 30 minutes on ice and centrifuged for 10 minutes at 10000 rpm at +4°C. In the supernatants the amount of proteins was determined according to the Bradford method (using BSA as a standard). The protein composition was analysed by SDS-PAGE.

F. Washing of inclusion bodies with 8 M urea

The determined quantity of inclusion bodies was resuspended in a 10-fold volume of cold (+4°C) buffer X (10 mM Tris/HCl, pH=8.0) with added 8 M urea, left for 30 minutes on ice and centrifuged for 10 minutes at 10000 rpm at +4°C. In the supernatants the amount of proteins was determined according to the Bradford method (using BSA as a standard). The protein composition was analysed by SDS-PAGE.

As shown in Figure 2, the solubility of the inclusion bodies was more increased when the cells were cultivated at 25°C (lanes 4–10) than when the inclusion bodies were cultivated at 37°C (lanes: 12–18, 20). When the cells were cultivated at 25°C, washing with the detergents (1% deoxycholate, 1% Triton-X100) and with 2 M urea was no longer possible as the majority of heterologous protein was lost with the washing solution (lanes 8, 9, 10), in the case the cells were cultivated at 37°C washing with the detergents (1% deoxycholate, lane 16), 2 M urea (lane 17) and 1% Triton-X100 (lane 18) was still possible. As seen, the inclusion bodies

obtained at the cultivation temperature 37°C could be solubilised only with 8 M urea (lane 20).

Example 4: Influence of the performing of fermentation on accumulation of G-CSF and on proportion of correctly folded precursor of G-CSF in inclusion bodies

High proportion of correctly folded precursor of G-CSF in inclusion bodies can be obtained by performing the fermention in a batch mode or fed-batch mode.

Performing fermentation as a batch mode

E. coli culture BL21(DE3) pET3a/P-Fopt5 from the strain bank at -70^0C was inoculated in a ratio 1 to 500 to LBG/amp medium and the culture was cultivated 14–18 hours on a shaker at 25°C, 150 rpm. This culture was used as an inoculation culture to inoculate the fermentor culture in a ratio 1 to 20 in the production medium GYST/amp to which the IPTG inductor to the final concentration of 0.4 mM was added at the beginning of the fermentation or in the early exponential phase when $OD_{\lambda 600nm}$ of the culture was 6. The fermentation was performed in a batch mode 18–25 hours at 25°C, 500 rpm and air flow 1 vvm (7 L Chemap fermentor). At the end of the process the $OD_{\lambda 600nm}$ of the culture was approximately 25 at the beginning of induction and 30 in the case of later induction. The biomass was centrifuged for 5 minutes at 5000 rpm (Beckman centrifuge) and frozen for the further processing. After SDS-PAGE and staining with Coomassie, the proportion of G-CSF in total proteins was determined by using a densitometric analysis. It ranged between 30% and 40%.

Performing of fermentation in a fed-batch mode

The fermentation was performed in a fed-batch mode 25.5–30 hours at 25°C, 500 rpm and air flow 1 vvm (7 L Chemap fermentor). Once the glucose in the medium was consumed, that is, at the end of the batch process (performed as described above), and the pH of the medium increased, feeding a 20% glucose solution with 100 mg/l ampicillin was initiated to provide the specific growth rate μ between 0.05 and 0.1 hr^{-1}. The process was completed after 7–7.5 hours of feeding (totally 25.5–30 hours) when the $OD_{\lambda 600nm}$ of the culture was approximately 42. The

biomass was centrifuged for 5 minutes at 5000 rpm (Beckman centrifuge) and frozen for further processing. After SDS-PAGE and staining with Coomassie, accumulation of G-CSF, maintained at a similar high level as in the case of performing the fermentation in a batch mode, was determined by using the batch method, was determined by using a densitometric analysis. It ranged between 30% and 40%. The inclusion bodies were isolated from the biomass, as described in Example 3. The pellet with inclusion bodies was washed with cold water, centrifuged again for 30 minutes at 10000 rpm and solubilised under non-denaturing conditions in a 50-fold the volume of 0.2 % N-lauroyl sarcosine in 40 mM TrisHCl/pH=8.0. Solubilisation was left to run overnight for 16 to 18 hours at room temperature under shaking at 100–150 rpm. After solubilisation the concentration of proteins was determined by the Bradford method using pure hMet-G-CSF as a standard.

Concentrations of the proteins ranged between 2 and 4 mg/ml after both principles of performing the fermentation. The proportion of correctly folded precursor of G-CSF or biologically active G-CSF was determined by measuring biologically activity of the solubilised inclusion bodies without removal of N-lauroyl sarcosine (at this dilution sarcosyl does not interfere with).

Principle of performing the fermentation	Induction with 0.4 mM IPTG	Proportion of biologically active G-CSF
batch	at the beginning	44%
fed-batch	at the beginning	39%
batch	$OD_{600nm} \approx 6$	43%
fed-batch	$OD_{600nm} \approx 6$	37%

Table 2: Proportion of correctly folded precursor of G-CSF regarding to the way of performing the fermentation and the induction mode

As evident from the data presented in Table 2, the high proportion of the biologically active G-CSF was obtained in all cases, being the highest by performing the fermentation in a batch mode.

Example 5: Influence of the induction with lactose on accumulation of G-CSF and proportion of correctly folded precursor of G-CSF

E. coli culture BL21(DE3) pET3a/P-Fopt5 from the strain bank at -70^0C was inoculated in a ratio 1 to 500 to LBG/amp medium and the culture was cultivated

14–18 hours on a shaker at 25°C, 150 rpm. This culture was used as an inoculation culture to inoculate the fermentor culture in a ratio 1 to 20 in the modified production medium LYST/amp wherein glycerol (6 g/l) and lactose (2 g/l or 4 g/l) were used instead of glucose as the source of carbon which simultaneously were also the expression inductor instead of IPTG. The fermentation was performed as a batch mode for 17–21 hours at 25°C, 500 rpm and air flow 1 vvm (7 L Chemap fermentor). At the end of the process the $OD_{\lambda 600nm}$ of the culture was approximately 20. The biomass was centrifuged for 5 minutes at 5000 rpm (Beckman centrifuge), washed 1x with 10 mM Tris/HCl, pH=8 buffer and frozen for the further processing. After SDS-PAGE and staining with Coomassie, accumulation of G-CSF was determined by using a densitometric analysis, which was 27% for induction with 2 g/l lactose and 33% for induction with 4g/l lactose. The inclusion bodies were isolated from the biomass, as described in Example 3. The pellet with inclusion bodies was washed with cold water, centrifuged again for 30 minutes at 10000 rpm and solubilised under non-denaturing conditions in a 50-fold the volume of 0.2 % N-lauroyl sarcosine in 40 mM TrisHCl/pH=8.0. Solubilisation was left overnight for 16 to 18 hours at room temperature under shaking at 100–150 rpm.

Expression system	Induction with lactose	Cultivation temperature	Proportion of biologically active G-CSF
pET3a / P-Fopt5 *E. coli* BL21 (DE3)	2 g/l	25°C	≈ 25%
pET3a / P-Fopt5 *E. coli* BL21 (DE3)	4 g/l	25°C	≈ 23%

Table 3: Proportion of correctly folded precursor of G-CSF or proportion of biologically active G-CSF (in %) in the inclusion bodies obtained by induction of the production strain *E. coli* BL21 (DE3) pET3a/P-Fopt5 with lactose.

As evident from Table 3, using the induction with lactose over a 23% proportion of the correctly folded precursor of G-CSF in inclusion bodies was obtained when cultivated at 25°C.

Example 6: Influence of the induction with NaCl on accumulation of G-CSF and on proportion of correctly folded precursor of G-CSF

E. coli culture BL21(DE3) pET3a-Fopt5 from the strain bank at -70^0C was inoculated in a ratio 1 to 20 in the LBON/amp medium and cultivated over the day for 8 hours on a shaker at 25°C, 160 rpm. 1 ml of this culture was used as an inoculation culture to inoculate 20 ml of GYSPON/amp 200 medium to which the NaCl inductor to the final concentration of 1.2 M was added at the beginning or at the $OD_{\lambda 600nm} \approx 0.5$, that is, after approximately 3 hours of cultivation. In both cases the culture was cultivated on a shaker at 25°C and 160 rpm for 20–24 hours. For SDS-PAGE analysis, after completed cultivation 5 ml of this culture was centrifuged for 5 minutes at 5000 rpm. The pellets were then resuspended in 15 ml of 10 mM TrisHCl/pH=8.0. The samples were mixed in a ratio 3 to 1 with 4x SDS – sample buffer with DTT (pH=8.7) and heated at 95°C for 10 minutes, centrifuged and the supernatant was loaded to the gel. Accumulation of G-CSF is presented in Table 4. As shown in the table, higher accumulation of G-CSF was obtained when 1.2 M NaCl was added to the medium at the beginning, that is, at inoculation and not conventionally at $OD_{\lambda 600nm} \approx 0.5$, as recommended by the producer (Life Technologies).

Expression system	Cultivation and induction conditions	Cultivation temperature	Accumulation of G-CSF
pET3a / P-Fopt5 *E. coli* BL21-SI	GYSPON/amp100 1.2 M NaCl at the beginning	25°C	25%
pET3a / P-Fopt5 *E. coli* BL21-SI	GYSPON/amp100 1.2 M NaCl at $OD_{\lambda 600nm} \approx 0.5$,	25°C	17%

Table 4: Comparison of accumulation of G-CSF of the production strain BL21-SI pET3a/P-Fopt5 in different media and induction modes.

The values reported for the G-CSF assay were obtained by the densitometric analysis of SDS-PAGE gels stained with Coomassie brilliant blue (Figure 3). Relative proportion was determined by the profile analysis (program Molecular analyst; BioRad) of gels on the apparatus Imaging densitometer Model GS670 (BioRad).

Preparation of the samples for determination of biological activity and inclusion bodies washing experiments

For the analysis of biological activity and inclusion bodies washing experiments a larger quantity of biomass was prepared. *E. coli* culture BL21-SI pET3a-Fopt5 from the strain bank at –70°C was inoculated in a ratio 1 to 20 in the LBON/amp medium and cultivated over the day for 8 hours on a shaker at 25°C, 160 rpm. 10- ml aliquots of this culture were incubated in 8 x 200 ml of GYSPON/amp 200 medium to which the NaCl inductor to the final concentration of 1.2 M was added already at the beginning. The culture was cultivated on a shaker at 25°C and 160 rpm for 24 hours. The inclusion bodies were isolated from the biomass, as described in Example 3. The pellet with inclusion bodies was washed with cold water and solubilised with 0.2 % N-lauroyl sarcosine as described in Example 9. After solubilisation, the protein concentration was determined according to the Bradford method using pure hMet-G-CSF as a standard. The concentration of total solubilised proteins ranged between 2 and 3 mg/ml.

Expression system	Cultivation temperature	Proportion of biologically active G-CSF
pET3a / P-Fopt5 *E. coli* BL21-SI	25°C	≈ 40%

Table 5: Proportion of correctly folded precursor of G-CSF or biologically active G-CSF in inclusion bodies obtained by the induction of a production strain *E. coli* BL21-SI pET3a / P-Fopt5 with 1.2 M NaCl.

As evident from Table 5, a high proportion of correctly folded precursor of G-CSF (or biologically active G-CSF) was obtained with a production strain *E. coli* BL21-SI pET3a/P-Fopt5 by induction of the expression of heterologous gene with 1.2 M NaCl when cultivated at 25°C.

Solubility of inclusion bodies

The solubility of inclusion bodies was much increased with a production strain *E. coli* BL21-SI pET3a/P-Fopt5 by induction of the expression of the heterologous gene with 1.2 M NaCl when cultivated at 25°C. Here, too, washing of the inclusion

bodies with detergents would lead to losses of the correctly folded precursor of G-CSF in the washing solution (Figure 4).

Example 7: Influence of the additives capable of causing stress, e.g., ethanol or propanol, on proportion of correctly folded precursor of G-CSF in inclusion bodies

E. coli culture BL21(DE3) pET3a-Fopt5 from the strain bank at –70°C was inoculated in a ratio 1 to 500 in the LBPG/amp100 medium (10 g/l phytone, 5 g/l yeast extract, 10 g/l NaCl, 100 mg/ml ampicillin) and cultivated for 17 hours on a shaker at 25°C, 160 rpm. 10 ml of this culture was inoculated in 200 ml of the medium with instantaneous addition of the IPTG inductor to the final concentration of 0.4 mM:

- GYSP/amp100 medium (control);
- GYSP/amp100 medium with added ethanol (final ethanol concentration in the medium 3%);
- GYSP/amp100 medium with added iso-propanol (final iso-propanol concentration in the medium 3%).

The culture was then cultivated on a shaker at 25°C and 160 rpm for 24 hours in the case of the control (GYSP/amp100 medium). In the case of GYSP/amp100 medium with added ethanol or iso-propanol, the cultures were cultivated under the same conditions for 34 hours, as addition of the either additive slowed the growth . inclusion bodies were isolated from the biomass, as described in Example 3. The pellet containing the inclusion bodies was washed with cold water and solubilised with addition of 0.2% N-lauroyl sarcosine as described in Example 9.

Expression system	Cultivation temperature in °C	Additive	Proportion of biologically active G-CSF
pET3a / P-Fopt5 *E. coli* BL21 (DE3)	25°C	Without additive	≈ 50%
pET3a / P-Fopt5 *E. coli* BL21 (DE3)	25°C	3% ethanol	≈ 59%
pET3a / P-Fopt5 *E. coli* BL21 (DE3)	25°C	3 % propanol	≈ 62%

Table 6: Proportion of correctly folded precursor of G-CSF (in %) in inclusion bodies obtained by the induction of a production strain *E. coli* BL21 (DE3) pET3a / P-Fopt5 in GYSP/amp100 medium with 0.4 mM IPTG and with addition of ethanol or propanol which are capable of causing stress.

As evident from Table 6, the additions of ethanol or propanol, which trigger stress proteins, increase the proportion of correctly folded precursor of G-CSF or biologically active G-CSF in inclusion bodies.

Example 8: Solubilisation of inclusion bodies with 2 M urea in buffer Z (40mM Tris/HCl, pH 8.0)

12 ml of buffer Z 40 mM Tris/HCl pH=8.0) with 2 M urea was added to 0.30 g of inclusion bodies which were previously washed with cold water (see Example 3). After homogenisation the inclusion bodies were solubilised for 16 hours at 20°C under gentle shaking at 80 rpm. The solution was centrifuged for 10 minutes at 14000 rpm and +10°C and the pellet was thrown away. The supernatant containing inclusion bodies was used for determination of the total protein content, SDS-PAGE analyses and for determination of biological activity.

The protein concentration according to the Bradford method (using hMetG-CSF as a standard): 2.6 mg/ml, total solubilised proteins: 31 mg, (the amount of solubilised proteins: approx. 10% of inclusion bodies mass).

Biological activity of G-CSF: about 1.5×10^7 IU/mg

Example 9: Solubilization of inclusion bodies with 0.2% N-lauroyl-sarcosine in buffer Z (40 mM Tris/HCl, pH 8.0)

15.6 ml of buffer Z (40 mM Tris/HCl pH=8.0) with 0.2% N-lauroyl-sarcosine was added to 0.31 g of inclusion bodies which were previously washed with cold water (see Example 3). After homogenisation the inclusion bodies were solubilised for 1 hour at room temperature (20–22°C) under shaking at 100–50 rpm. 0.1 M $CuSO_4 \times 5H_2O$ to the final concentration of 40 μM was added to promote oxidation (i.e. disulphide bonds formation). Shaking was left overnight (16 hours) at 80 rpm and at 20°C. Next day the solution was centrifuged for 10 minutes at 14000 rpm and +10°C and the pellet was thrown away. N-lauroyl-sarcosine was removed from the

supernatant containing the solubilised inclusion bodies by using DOWEX (DOWEX 1 Sigma), in such a way that 0.39 g DOWEX 1 was added, the solution was shaken for 1 hour at room temperature (20–22°C) and 100-150 rpm. The protein solution was then thrown away. The solution of solubilised inclusion bodies was used for determination of total protein content, SDS-PAGE analysis and for determination of biological activity. Protein concentration according to the Bradford method (using hMetG-CSF as a standard): 4.4 mg/ml, total solubilised protein content: 68 mg, (the amount of solubilised proteins; approx. 20% of inclusion bodies mass).

Biological activity of G-CSF: about 4.0 – 4.5 $x10^7$ IU/mg – independent of adding $CuSO_4 x\ 5H_2O$.

Example 10: Solubilisation of inclusion bodies with 0.2% NDSB (195, 201, 211, 256) in buffer Z (40 mM Tris/HCl, pH 8.0)

A 40-fold overage of buffer Z (40 mM Tris/HCl pH=8.0) with addition of different NDSB (non-detergent sulfobetaine) at the concentration of 0.2% was added to 0.16-g aliquots of inclusion bodies which were previously washed with cold water (see Example 3). NDSB 195, NDSB 201, NDSB 211 and NDSB 256 were used. After homogenisation, the samples of inclusion bodies were solubilised at 20°C under shaking at 80 rpm overnight. Simultaneously, the samples of inclusion bodies samples to which 0.1 M $CuSO_4 x\ 5H_2O$ to the final concentration of 40 μM was added after initial 30-minute solubilisation to promote oxidation were also solubilised, and shaking was continued overnight. Next day after centrifugation, the precipitates were thrown away, and the supernatants, without any prior processing, were used for determination of total protein content, SDS-PAGE analysis and for determination of biological activity. The protein concentration according to the Bradford method (using hMetG-CSF as a standard): about 3.6 mg/ml in all cases, thus, the total protein content: about 24 mg, (the amount of solubilised proteins: approx. 15% of inclusion bodies mass).

Biological activity of G-CSF: about 3 – 4 $x10^7$ IU/mg – independent of adding $CuSO_4 x\ 5H_2O$.

Example 11: Solubilisation of inclusion bodies with 5% DMSO in buffer Z (40mM Tris/HCl, pH 8.0)

A 40-fold overage of buffer Z (40 mM Tris/HCl pH=8.0) with 5% DMSO was added to 0.16 g of inclusion bodies which were previously washed with cold water (see Example 3). After homogenisation, inclusion bodies were solubilised at 20°C under shaking at 80 rpm overnight. Simultaneously, the sample of inclusion bodies to which to which 0.1 M $CuSO_4 \times 5H_2O$ to the final concentration of 40 μM was added after initial 30-minute solubilisation to promote oxidation was also solubilised, and shaking was continued overnight. Next day after centrifugation the precipitates were thrown away, and the supernatants, without any prior processing, were used for determination of total protein content, SDS-PAGE analysis and for determination of biological activity. The protein concentration according to the Bradford method (using hMetG-CSF as a standard): about 3.6 mg/ml, thus, the total protein content: about 24 mg, (the amount of solubilised proteins: approx. 15% of inclusion bodies mass).

Biological activity of G-CSF: about 4 $\times 10^7$ IU/mg – independent of adding $CuSO_4 \times 5H_2O$.

Example 12: Testing of biological activity of G-CSF *in vitro*

The biological activity of G-CSF was determined by using the proliferation assay on the cell line NFS-60 according to the known method (Hammerling U and co-workers in *J Pharm Biomed Anal* **13**, 9-20 (1995)) and use of the international standard Human recombinant G-CSF (88/502, yeast cell derived; NIBSC Potters Bar, Hertfordshire, UK); (Mire-Sluis A R and co-workers in *J Immunol Methods* **179**, 117-126 (1995)).

Patent claims

1. The process for production of proteins, wherein the correctly folded precursor of the heterologous protein after expression is accumulated in inclusion bodies.
2. The process for production of proteins according to claim 1, wherein the heterologous protein is selected from the group comprising: G-CSF, GM-CSF, M-CSF, EGF, HSA, DNAse, FGF, TNF-alpha, TNF-beta, interferons and interleukins.
3. The process for production of proteins according to claim 1, wherein the selected heterologous protein is G-CSF.
4. The process for production of proteins according to claim 1, wherein the expression is performed in the organisms selected from the group comprising bacteria and yeast.
5. The process for production of proteins according to claim 4, wherein the expression is performed in a bacterium *E. coli*.
6. The process for production of proteins according to claim 1, wherein the process involves the way of performing the biosynthesis comprising one or more parameters which are selected from the group comprising: temperature of cultivation, composition of cultivation medium, induction mode, principle of performing the fermentation and co-expression of auxiliary proteins.
7. The way of performing the biosynthesis according to claim 6, wherein the temperature of cultivation is between 20°C and 30°C.
8. The way of performing the biosynthesis according to claim 7, wherein the temperature of cultivation is about 25°C.
9. The way of performing the biosynthesis according to claim 6, wherein the inductor is selected from the group comprising IPTG, lactose and NaCl.
10. The way of performing the biosynthesis according to claim 9, wherein the selected inductor is IPTG.
11. The way of performing the biosynthesis according to claim 10, wherein the concentration of IPTG is in the range from 0.1 mM to 1 mM.
12. The way of performing the biosynthesis according to claim 11, wherein the concentration of IPTG is about 0.4 mM.

13. The way of performing the biosynthesis according to claim 9, wherein the inductor is added at the beginning of the fermentation.

14. The way of performing the biosynthesis according to claim 6, wherein the principle of performing the biosynthesis is selected from the group comprising: performing of fermentation in a batch mode, performing of fermentation in a fed batch mode and fermentation in shake flasks.

15. The way of performing the biosynthesis according to claim 14, wherein the selected principle of performing the fermentation is a batch mode.

16. The way of performing the biosynthesis according to claim 6, wherein the medium is selected from the group comprising: GYST/amp100, GYSP/amp100, LYSP/amp100, LYST/amp100, LBON/amp100 and GYSPON/amp100.

17. The way of performing the biosynthesis according to claim 16 wherein the selected medium is GYST/amp100.

18. The way of performing the biosynthesis according to claim 6, wherein the additives which are capable of causing stress are selected from the group comprising ethanol and propanol.

19. The process for production of proteins according to claim 6, wherein the process further comprises washing of inclusion bodies.

20. The washing of inclusion bodies according to claim 19, wherein the washing is performed by using solutions selected from the group comprising Tris/HCl buffer, phosphate buffer, acetate buffer, citrate buffer and water.

21. The washing of inclusion bodies according to claim 20, wherein the concentration of the buffers is in the range from 1 mM to 10 mM.

22. The washing of inclusion bodies according to claim 19, wherein the selected solution is water.

23. The process for production of proteins according to claim 19 wherein the process further comprises solubilisation of inclusion bodies.

24. The solubilisation of inclusion bodies according to claim 23, wherein the agents for solubilisation are selected from the group of solvents comprising urea in non-denaturing concentrations (1–2 M), N-lauroyl sarcosine in non-denaturing concentrations (0.05–0.2% (m/v)), low concentrations of Zwittergents, non-detergent sulfobetains, betain, sarcosine, carbamoil sarcosine, taurine, DMSO

and higher concentrations of several buffers, selected from the group comprising: HEPES, HEPPS, MES, ACES, MES.

25. The solubilisation of inclusion bodies according to claim 24, wherein the selected solvent is N-lauroyl-sarcosine.

26. The solubilisation of inclusion bodies according to claim 25, wherein the concentration of N-lauroyl sarcosine is in the range from 0.1% to 0.25%.

27. The solubilisation of inclusion bodies according to claim 26, wherein the concentration of N-lauroyl sarcosine is about 0.2%.

28. The process for production of biologically active G-CSF, wherein the selected parameters for the way of performing the biosynthesis are:

- cultivation temperature: about 25°C
- cultivation medium composition: GYST/amp100
- performing of fermentation: batch mode
- induction: IPTG with the concentration about 0.4 mM
- induction mode: at the beginning of the fermentation

29. The process for production of biologically active G-CSF according to claim 28, wherein the process further comprises washing of inclusion bodies with water.

30. The process for production of biologically active G-CSF according to claim 29, wherein the process further comprises the solubilisation of inclusion bodies with N-lauroyl sarcosine with the concentration of about 0.2%.

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Abstract

The invention relates to a new process for the production of the biologically active heterologous protein. The process involves the performing of biosynthesis (changing conditions) in such a way that the process enables the accumulation of correctly folded precursor of the heterologous protein already in the inclusion bodies. The invention further comprises washing and solubilising of the inclusion bodies.

Figure 1

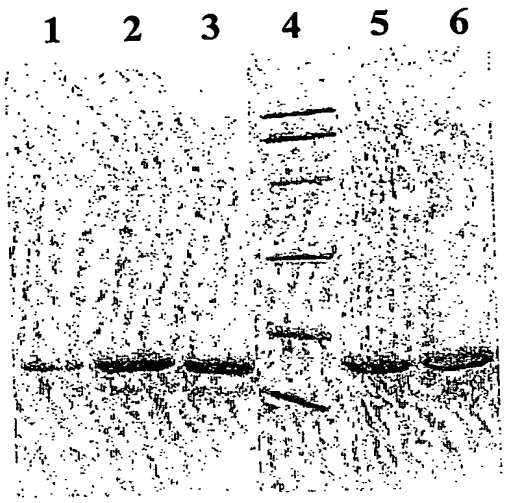

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Figure 2

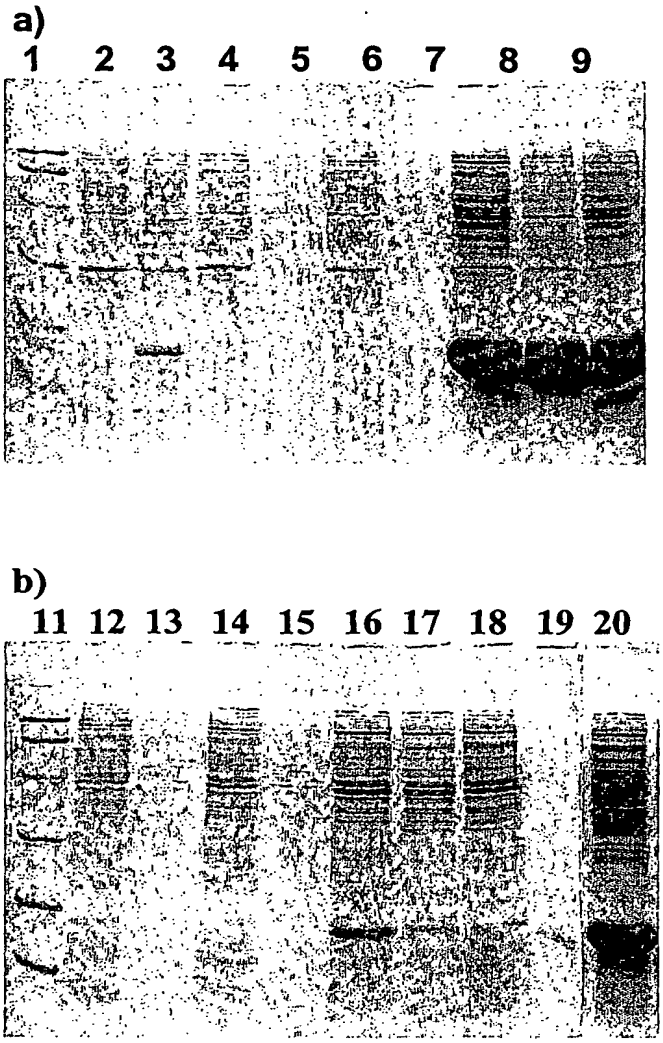

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Figure 3

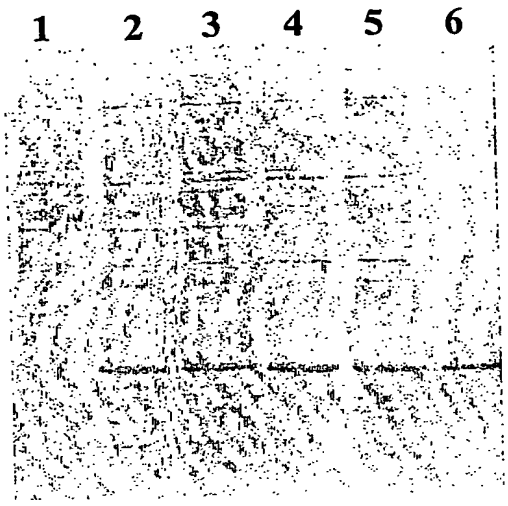

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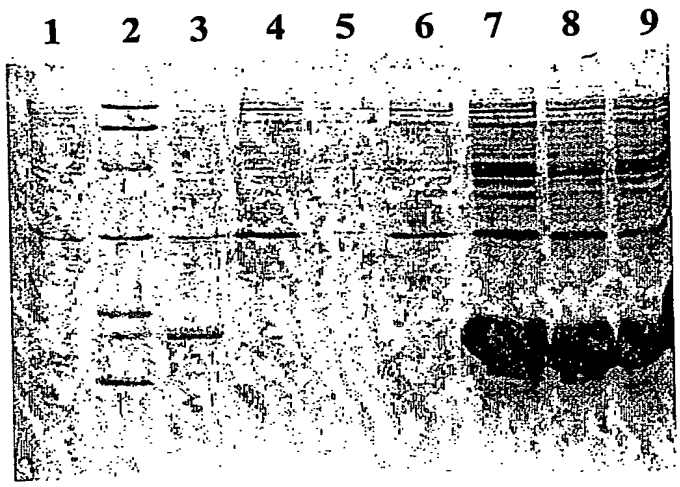
Figure 4



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The undersigned Djurdjica Mandrino, permanent court interpreter for the English language, appointed by Decree No. 756-4/91, issued on 11th of February 1991 by the Ministry of Justice and Administration, Republic of Slovenia, hereby declares that this document entirely corresponds to the original Slovene text.

Ljubljana, 12th March 2003